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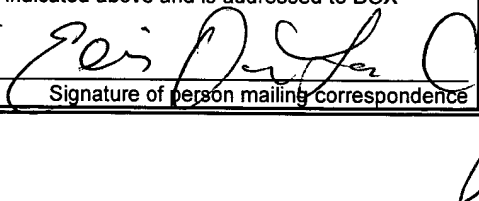
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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE : GRAPEVINE LEAFROLL VIRUS (TYPE 2)  
PROTEINS AND THEIR USES

## GRAPEVINE LEAFROLL VIRUS (TYPE 2) PROTEINS AND THEIR USES

5 This application claims the benefit of U.S. Provisional Patent Application  
Serial No. 60/047,194, filed May 20, 1997. This work was supported by the U.S. Department  
of Agriculture Cooperative Grant No. 58-2349-9-01. The U.S. Government may have certain  
rights in the invention.

### FIELD OF THE INVENTION

10 The present invention relates to grapevine leafroll virus (type 2) proteins,  
DNA molecules encoding these proteins, and their uses.

### BACKGROUND OF THE INVENTION

15 The world's most widely grown fruit crop, the grape (*Vitis sp.*), is cultivated  
on all continents except Antarctica. However, major grape production centers are in  
European countries (including Italy, Spain, and France), which constitute about 70% of the  
world grape production (Mullins et al., Biology of the Grapevine, Cambridge,  
U.K.:University Press (1992)). The United States, with 300,000 hectares of grapevines, is the  
20 eighth largest grape grower in the world. Although grapes have many uses, a major portion  
of grape production (~80%) is used for wine production. Unlike cereal crops, most of the  
world's vineyards are planted with traditional grapevine cultivars, which have been  
perpetuated for centuries by vegetative propagation. Several important grapevine virus and  
virus-like diseases, such as grapevine leafroll, corky bark, and *Rupestris* stem pitting, are  
25 transmitted and spread through the use of infected vegetatively propagated materials. Thus,  
propagation of certified, virus-free materials is one of the most important disease control  
measures. Traditional breeding for disease resistance is difficult due to the highly  
heterozygous nature and outcrossing behavior of grapevines, and due to polygenic patterns of  
inheritance. Moreover, introduction of a new cultivar may be prohibited by custom or law.  
30 Recent biotechnology developments have made possible the introduction of special traits,  
such as disease resistance, into an established cultivar without altering its horticultural  
characteristics.

Many plant pathogens, such as fungi, bacteria, phytoplasmas, viruses, and  
nematodes can infect grapes, and the resultant diseases can cause substantial losses in  
35 production (Pearson et al., Compendium of Grape Diseases, American Phytopathological

Society Press (1988)). Among these, viral diseases constitute a major hindrance to profitable growing of grapevines. About 34 viruses have been isolated and characterized from grapevines. The major virus diseases are grouped into: (1) the grapevine degeneration caused by the fanleaf nepovirus, other European nepoviruses, and American nepoviruses, (2) the leafroll complex, and (3) the rugose wood complex (Martelli, ed., Graft Transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, FAO, UN, Rome, Italy (1993)).

Of the major virus diseases, the grapevine leafroll complex is the most widely distributed throughout the world. According to Goheen ("Grape Leafroll," in Frazier et al., eds., Virus Diseases of Small Fruits and Grapevines (A Handbook), University of California, Division of Agricultural Sciences, Berkeley, Calif, USA, pp. 209-212 (1970) ("Goheen (1970)"), grapevine leafroll-like disease was described as early as the 1850s in German and French literature. However, the viral nature of the disease was first demonstrated by Scheu (Scheu, "Die Rollkrankheit des Rebstockes (Leafroll of grapevine)," D. D. Weinbau 14:222-358 (1935) ("Scheu (1935)"). In 1946, Harmon and Snyder (Harmon et al., "Investigations on the Occurrence, Transmission, Spread and Effect of 'White' Fruit Colour in the Emperor Grape," Proc. Am. Soc. Hort. Sci. 74:190-194 (1946)) determined the viral nature of White Emperor disease in California. It was later proven by Goheen et al. (Goheen et al., "Leafroll (White Emperor Disease) of Grapes in California," Phytopathology, 48:51-54 (1958) ("Goheen (1958)")) that both leafroll and "White Emperor" diseases were the same, and only the name "leafroll" was retained.

Leafroll is a serious viral disease of grapes and occurs wherever grapes are grown. This wide distribution of the disease has come about through the propagation of diseased vines. It affects almost all cultivated and rootstock varieties of *Vitis*. Although the disease is not lethal, it causes yield losses and reduction of sugar content. Scheu estimated in 1936 that 80 per cent of all grapevines planted in Germany were infected (Scheu, Mein Winzerbuch, Berlin:Reichsnährstand-Verlags (1936)). In many California wine grape vineyards, the incidence of leafroll (based on a survey of field symptoms conducted in 1959) agrees with Scheu's initial observation in German vineyards (Goheen et al., "Studies of Grape Leafroll in California," Amer. J. Enol. Vitic., 10:78-84 (1959)). The current situation on leafroll disease does not seem to be any better (Goheen, "Diseases Caused by Viruses and Viruslike Agents," The American Phytopathological Society, St. Paul, Minnesota:APS Press, 1:47-54 (1988) ("Goheen (1988)"). Goheen also estimated that the disease causes an annual loss of about 5-20 per cent of the total grape production (Goheen (1970) and Goheen (1988)).

The amount of sugar in individual berries of infected vines is only about 1/2 to 2/3 that of berries from noninfected vines (Goheen (1958)).

Symptoms of leafroll disease vary considerably depending upon the cultivar, environment, and time of the year. On red or dark-colored fruit varieties, the typical downward rolling and interveinal reddening of basal, mature leaves is the most prevalent in autumn; but not in spring or early summer. On light-colored fruit varieties however, symptoms are less conspicuous, usually with downward rolling accompanied by interveinal chlorosis. Moreover, many infected rootstock cultivars do not develop symptoms. In these cases, the disease is usually diagnosed with a woody indicator indexing assay using *Vitis* 5 *vivifera* cv. Carbernet Franc (Goheen (1988)).

Ever since Scheu demonstrated that leafroll was graft transmissible, a virus etiology has been suspected (Scheu (1935)). Several virus particle types have been isolated from leafroll diseased vines. These include potyvirus-like (Tanne et al., "Purification and Characterization of a Virus Associated with the Grapevine Leafroll Disease," 10 *Phytopathology*, 67:442-447 (1977)), isometric virus-like (Castellano et al., "Virus-like Particles and Ultrastructural Modifications in the Phloem of Leafroll-affected Grapevines," *Vitis*, 22:23-39 (1983) ("Castellano (1983)"), and Namba et al., "A Small Spherical Virus Associated with the Ajinashika Disease of Koshu Grapevine, *Ann. Phytopathol. Soc. Japan*, 45:70-73 (1979)), and closterovirus-like (Namba, "Grapevine Leafroll Virus, a Possible Member of Closteroviruses, *Ann. Phytopathol. Soc. Japan*, 45:497-502 (1979)) particles. In recent years, however, long flexuous closteroviruses ranging from 1,400 to 2,200 nm have been most consistently associated with leafroll disease (Figure 1) (Castellano (1983), Faoro et al., "Association of a Possible Closterovirus with Grapevine Leafroll in Northern Italy," 20 *Riv. Patol. Veg., Ser IV*, 17:183-189 (1981), Gugerli et al., "L'enroulement de la vigne: mise en évidence de particules virales et développement d'une méthode immuno-enzymatique pour le diagnostic rapide (Grapevine Leafroll: Presence of Virus Particles and Development of an Immuno-enzyme method for Diagnosis and Detection)," *Rev. Suisse Viticult. Arboricult. Hort.*, 16:299-304 (1984) ("Gugerli (1984)"), Hu et al., "Characterization of Closterovirus-like Particles Associated with Grapevine Leafroll Disease," *J. Phytopathol.*, 128:1-14 (1990) ("Hu (1990)"), Milne et al., "Closterovirus-like Particles of Two Types Associated with Diseased Grapevines," *Phytopathol. Z.*, 110:360-368 (1984), Zee et al., "Cytopathology of Leafroll-diseased Grapevines and the Purification and Serology of Associated Closteroviruslike Particles," *Phytopathology*, 77:1427-1434 (1987) ("Zee (1987)"), and Zimmermann et al., "Characterization and Serological Detection of Four

Closterovirus-like Particles Associated with Leafroll Disease on Grapevine," J. Phytopathol., 130:205-218 (1990) ("Zimmermann (1990)"). These closteroviruses are referred to as grapevine leafroll associated viruses ("GLRaV"). At least six serologically distinct types of GLRaV's (GLRaV-1 to -6) have been detected from leafroll diseased vines (Table 1) (Boscia et al., "Nomenclature of Grapevine Leafroll-associated Putative Closteroviruses, Vitis, 34:171-175 (1995) ("Boscia (1995)") and (Martelli, "Leafroll," pp. 37-44 in Martelli, ed., Graft Transmissible Diseases of Grapevines, Handbook for Detection and Diagnosis, FAO, Rome Italy, (1993) ("Martelli I"). The first five of these were confirmed in the 10th Meeting of the International Council for the Study of Virus and Virus Diseases of the Grapevine ("ICVG") (Volos, Greece, 1990).

TABLE 1

Type	Particle length (nm)	Coat protein <i>Mr</i> (X10 <sup>3</sup> )	Reference
GLRaV-1	1,400-2,200	39	Gugerli (1984)
GLRaV-2	1,400-1,800	26	Gugerli (1984) Zimmermann (1990)
GLRaV-3	1,400-2,200	43	Zee (1987)
GLRaV-4	1,400-2,200	36	Hu (1990)
GLRaV-5	1,400-2,200	36	Zimmermann (1990)
GLRaV-6	1,400-2,200	36	Gugerli (1993)

Through the use of monoclonal antibodies, however, the original GLRaV II described in Gugerli (1984) has been shown to be an apparent mixture of at least two components, IIa and IIb (Gugerli et al., "Grapevine Leafroll Associated Virus II Analyzed by Monoclonal Antibodies," 11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the Grapevine, Montreux, Switzerland, pp. 23-24 (1993) ("Gugerli (1993)").

Recent investigation with comparative serological assays (Boscia (1995)) demonstrated that the IIb component of cv. Chasselas 8/22 is the same as the GLRaV-2 isolate from France (Zimmermann (1990)) which also include the isolates of grapevine corky bark associated closteroviruses from Italy (GCBaV-BA) (Boscia (1995)) and from the United States

(GCBaV-NY) (Namba et al., "Purification and Properties of Closterovirus-like Particles Associated with Grapevine Corky Bark Disease," Phytopathology, 81:964-970 (1991) ("Namba (1991)")). The IIa component of cv. Chasselas 8/22 was given the provisional name of grapevine leafroll associated virus 6 (GLRaV-6). Furthermore, the antiserum to the  
5 CA-5 isolate of GLRaV-2 produced by Boscia et al. (Boscia et al., "Characterization of Grape Leafroll Associated Closterovirus (GLRaV) Serotype II and Comparison with GLRaV Serotype III," Phytopathology, 80:117 (1990)) was shown to contain antibodies to both GLRaV-2 and GLRaV-1, with a prevalence of the latter (Boscia (1995)).

Virions of GLRaV-2 are flexuous, filamentous particles about 1,400-1,800 nm  
10 in length (Gugerli et al., "L'enroulement de la Vigne: Mise en Evidence de Particles Virales et Developement d'une Methode Immuno-enzymatique Pour le Diagnostic Rapide (Grapevine Leafroll: Presence of Virus Particles and Development of an Immuno-enzyme Method for Diagnosis and Detection)," Rev. Suisse Viticult. Arboricult. Horticult. 16:299-304 (1984)). A double-stranded RNA (dsRNA) of about 15 kb was consistently isolated from GLRaV-2  
15 infected tissues (Goszczynski et al., "Detection of Two Strains of Grapevine Leafroll-Associated Virus 2," Vitis 35:133-35 (1996)). The coat protein of GLRaV-2 is ca 22~26 kDa (Zimmermann et al., "Characterization and Serological Detection of Four Closterovirus-like Particles Associated with Leafroll Disease on Grapevine," J. Phytopathology 130:205-18 (1990); Gugerli and Ramel, Extended abstracts: "Grapevine Leafroll Associated Virus II  
20 Analyzed by Monoclonal Antibodies," 11th ICVG at Montreux, Switzerland, Gugerli, ed., Federal Agricultural Research Station of Changins, CH-1260 Nyon, Switzerland, p. 23-24 (1993); Boscia et al., "Nomenclature of Grapevine Leafroll-Associated Putative Closteroviruses," Vitis 34:171-75 (1995)), which is considerably smaller than other GLRaVs (35~43 kDa) (Zee et al., "Cytopathology of Leafroll-Diseased Grapes and the  
25 Purification and Serology of Associated Closterovirus Like Particles," Phytopathology 77:1427-34 (1987); Hu et al., "Characterization of Closterovirus-Like Particles Associated with Grapevine Leafroll Disease," J. of Phytopathology 128:1-14 (1990); Ling et al., "The Coat Protein Gene of Grapevine Leafroll Associated Closterovirus-3: Cloning, Nucleotide  
30 Sequencing and Expression in Transgenic Plants," Arch. of Virology 142:1101-16 (1997)). Although GLRaV-2 has been classified as a member of the genus *Closterovirus* based on particle morphology and cytopathology (Martelli, Circular of ICTV-Plant Virus Subcommittee Study Group on Closterolike Viruses" (1996)), its molecular and biochemical properties are not well characterized.

- In the closterovirus group, several viruses have recently been sequenced. The partial or complete genome sequences of beet yellows virus (BYV) (Agranovsky et al. "Nucleotide Sequence of the 3'-Terminal Half of Beet Yellows Closterovirus RNA Genome Unique Arrangement of Eight Virus Genes," J. General Virology 72:15-24 (1991);
- 5 Agranovsky et al., "Beet Yellows Closterovirus: Complete Genome Structure and Identification of a Papain-like Thiol Protease," Virology 198:311-24 (1994)), beet yellow stunt virus (BYSV) (Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses," Virology 221:199-207 (1996)), citrus tristeza virus (CTV) (Pappu et al., "Nucleotide Sequence and
- 10 Organization of Eight 3' Open Reading Frames of the Citrus Tristeza Closterovirus Genome," Virology 199:35-46 (1994); Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," Virology 208:511-20 (1995)), lettuce infectious yellows virus (LIYV) (Klaassen et al., "Partial Characterization of the Lettuce Infectious Yellows Virus Genomic RNAs, Identification of the Coat Protein Gene and Comparison of its Amino Acid Sequence
- 15 With Those of Other Filamentous RNA Plant Viruses," J. General Virology 75:1525-33 (1994); Klaassen et al., "Genome Structure and Phylogenetic Analysis of Lettuce Infectious Yellows Virus, a Whitefly-Transmitted, Bipartite Closterovirus," Virology 208:99-110 (1995)), little cherry virus (LChV) (Keim and Jelkmann, "Genome Analysis of the 3'-
- 20 Terminal Part of the Little Cherry Disease Associated dsRNA Reveals a Monopartite Clostero-Like Virus," Arch. Virology 141:1437-51 (1996); Jelkmann et al., "Complete Genome Structure and Phylogenetic Analysis of Little Cherry Virus, a Mealybug-Transmissible Closterovirus," J. General Virology 78:2067-71 (1997)), and GLRaV-3 (Ling et al., "Nucleotide Sequence of the 3' Terminal Two-Thirds of the Grapevine Leafroll Associated Virus-3 Genome Reveals a Typical Monopartite Closterovirus," J. Gen. Virology
- 25 79(5):1289-1301 (1998)) revealed several common features of the closteroviruses, including the presence of HSP70 chaperone heat shock protein and a duplicate of the coat protein gene (Agranovsky "Principles of Molecular Organization, Expression, and Evolution of Closteroviruses: Over the Barriers," Adv. in Virus Res. 47:119-218 (1996); Dolja et al.
- 30 "Molecular Biology and Evolution of Closteroviruses: Sophisticated Build-up of Large RNA Genomes," Annual Rev. Photopathology 32:261-85 (1994); Boyko et al., "Coat Protein Gene Duplication in a Filamentous RNA Virus of Plants," Proc. Nat. Acad. Sci. USA 89:9156-60 (1992)). Characterization of the genome organization of GLRaVs would provide molecular information on the serologically distinct closteroviruses that cause similar leafroll symptoms in grapevine.

Several shorter closteroviruses (particle length 800 nm long) have also been isolated from grapevines. One of these, called grapevine virus A ("GVA") has also been found associated, though inconsistently, with the leafroll disease (Agran et al., "Occurrence of Grapevine Virus A (GVA) and Other Closteroviruses in Tunisian Grapevines Affected by Leafroll Disease," Vitis, 29:43-48 (1990), Conti, et al., "Closterovirus Associated with Leafroll and Stem Pitting in Grapevine," Phytopathol. Mediterr., 24:110-113 (1985), and Conti et al., "A Closterovirus from a Stem-pitting-diseased Grapevine," Phytopathology, 70:394-399 (1980)). The etiology of GVA is not really known; however, it appears to be more consistently associated with rugose wood *sensu lato* (Rosciglione et al., "Maladies de l'enroulement et du bois strié de la vigne: analyse microscopique et sérologique (Leafroll and Stem Pitting of Grapevine: Microscopical and Serological Analysis)," Rev. Suisse Vitic Arboric. Hortic., 18:207-211 (1986) ("Rosciglione (1986)"), and Zimmermann (1990)). Moreover, another short closterovirus (800 nm long) named grapevine virus B ("GVB") has been isolated and characterized from corky bark-affected vines (Boscia et al., "Properties of a Filamentous Virus Isolated from Grapevines Affected by Corky Bark," Arch. Virol., 130:109-120 (1993) and Namba (1991)).

As suggested by Martelli I, leafroll symptoms may be induced by more than one virus or they may be simply a general plant physiological response to invasion by an array of phloem-inhabiting viruses. Evidence accumulated in the last 15 years strongly favors the idea that grapevine leafroll is induced by one (or a complex) of long closteroviruses (particle length 1,400 to 2,200 nm).

Grapevine leafroll is transmitted primarily by contaminated scions and rootstocks. However, under field conditions, several species of mealybugs have been shown to be the vector of leafroll (Engelbrecht et al., "Transmission of Grapevine Leafroll Disease and Associated Closteroviruses by the Vine Mealybug Planococcus-ficus," Phytophylactica, 22:341-346 (1990), Rosciglione, et al., "Transmission of Grapevine Leafroll Disease and an Associated Closterovirus to Healthy Grapevine by the Mealybug Planococcus ficus," (Abstract), Phytoparasitica, 17:63-63 (1989), and Tanne, "Evidence for the Transmission by Mealybugs to Healthy Grapevines of a Closter-like Particle Associated with Grapevine Leafroll Disease," Phytoparasitica, 16:288 (1988)). Natural spread of leafroll by insect vectors is rapid in various parts of the world. In New Zealand, observations of three vineyards showed that the number of infected vines nearly doubled in a single year (Jordan et al., "Spread of Grapevine Leafroll and its Associated Virus in New Zealand Vineyards," 11th Meeting of the International Council for the Study of Viruses and Virus Diseases of the



Grapevine, Montreux, Switzerland, pp. 113-114 (1993)). One vineyard became 90% infected 5 years after GLRaV-3 was first observed. Prevalence of leafroll worldwide may increase as chemical control of mealybugs becomes more difficult due to the unavailability of effective insecticides.

5 In view of the serious risk grapevine leafroll virus poses to vineyards and the absence of an effective treatment of it, the need to prevent this affliction continues to exist. The present invention is directed to overcoming this deficiency in the art.

### SUMMARY OF INVENTION

10 The present invention relates to an isolated protein or polypeptide corresponding to a protein or polypeptide of a grapevine leafroll virus (type 2). The encoding RNA and DNA molecules, in either isolated form or incorporated in an expression system, a host cell, a transgenic *Vitis* or *citrus* scion or rootstock cultivar, or a transgenic *Nicotiana*  
15 plant or beet plant are also disclosed.

Another aspect of the present invention relates to a method of imparting grapevine leafroll virus (type 2) resistance to *Vitis* scion or rootstock cultivars or *Nicotiana* plants by transforming them with a DNA molecule encoding the protein or polypeptide corresponding to a protein or polypeptide of a grapevine leafroll virus (type 2). Other aspects  
20 of the present invention relate to a method of imparting beet yellows virus resistance to beet plants and a method of imparting tristeza virus resistance to citrus scion or rootstock cultivars, both by transforming the plants or cultivars with a DNA molecule encoding the protein or polypeptide corresponding to a protein or polypeptide of a grapevine leafroll virus (type 2).

25 The present invention also relates to an antibody or binding portion thereof or probe which recognizes the protein or polypeptide.

Grapevine leafroll virus resistant transgenic variants of the current commercial grape cultivars and rootstocks allows for more complete control of the virus, while retaining the varietal characteristics of specific cultivars. Furthermore, these variants permit control of  
30 GLRaV-2 transmitted either by contaminated scions or rootstocks or by a presently uncharacterized insect vector. With respect to the latter mode of transmission, the present invention circumvents increased restriction of pesticide use which has made chemical control of insect infestation increasingly difficult. In this manner, the interests of the environment

and the economics of grape cultivation and wine making are all furthered by the present invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

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Figures 1A and 1B are a comparison of a double-stranded RNA (dsRNA) profile (Figure 1A) of GLRaV-2 and its Northern hybridization analysis (Figure 1B). In Figure 1A: lane M, lambda Hind III DNA marker; and lane 1, dsRNA pattern in 1% agarose gel stained with ethidium bromide. Figure 1B is a northern hybridization of isolated high molecular weight dsRNA of GLRaV-2 with a probe prepared with  $^{32}\text{P}$  [ $\alpha$ -dATP] labeled cDNA insert from GLRaV-2 specific cDNA clone TC-1. Lane 1, high molecular weight dsRNA of GLRaV-2. Lane 2, total RNA extracted from healthy grapevine.

Figure 2 displays the genome organization of GLRaV-2 and its sequencing strategy. Boxes represent ORFs encoded by deduced amino acid sequences of GLRaV-2, numbered lines represent nucleotide coordinates, beginning from 5'-terminal of RNA in kilobases (kb). The lines below GLRaV-2 RNA genome represent the cDNA clones used to determine the nucleotide sequences.

Figure 3A-3D are comparisons between ORF1a/ORF1b of GLRaV-2 and BYV. Figure 3A-3D show the conserved domains of two papain-like proteases (P-PRO), methyltransferase (MT/MTR), helicase (HEL), and RNA-dependent RNA polymerase (RdRP), respectively. Exclamation marks indicate the predicted catalytic residues of the leader papain-like protease; slashes indicate the predicted cleavage sites. The conserved motifs of the MT, HEL, and RdRP domains are highlighted with overlines marked with respective letters. The alignment is constructed using the MegAlign program in DNASTAR.

Figures 4A and 4B are alignments of the nucleotide (Figure 4A) and deduced amino acid (Figure 4B) sequences of ORF1a/ORF1b overlapping region of GLRaV-2, BYV, BYSV, and CTV. Identical nucleotides and amino acids are shown in consensus. GLRaV-2 putative + 1 frameshift site (TAGC) and its corresponding sites of BYV (TAGC) and BYSV (TAGC) and CTV (CGGC) at nucleotide and amino acid sequences are highlighted with underlines.

Figure 5 is an alignment of the amino acid sequence of HSP70 protein of GLRaV-2 and BYV. The conserved motifs (A to H) are indicated with overlines and marked

with respective letters. The alignment was conducted with the MegAlign program of DNASTAR.

Figure 6A is a comparison of the coat protein (CP) and coat protein duplicate (CPd) of GLRaV-2 with other closteroviruses. The amino acid sequence of the GLRaV-2 CP and CPd are aligned with the CP and CPd of BYV, BYSV, and CTV. The conserved amino acid residues are in bold and the consensus sequences are indicated. Sequence alignment and phylogenetic tree were constructed by Clustal Method in the MegAlign Program of DNASTAR. Figure 6B is a tentative phylogenetic tree of the CP and CPd of GLRaV-2 with BYV, BYSV, CTV, LIYV, LChV, and GLRaV-3. To facilitate the alignment, only the C-terminal 250 amino acids of CP and CPd of LIYV, LChV, and GLRaV-3 were used. The scale beneath the phylogenetic tree represents the distance between sequences. Units indicate the number of substitution events.

Figure 7 is a comparison of the genome organization of GLRaV-2, BYV, BYSV, CTV, LIYV, LChV, and GLRaV-3. P-PRO, papain-like protease; MT/MTR, methyltransferase; HEL, helicase; RdRP, RNA-dependent RNA polymerase; HSP70, heat shock protein 70; CP, coat protein; CPd, coat protein duplicate.

Figure 8 is a tentative phylogenetic tree showing the relationship of RdRP of GLRaV-2 with respect to BYV, BYSV, CTV, and LIYV. The phylogenetic tree was constructed using the Clustal method with the MegAlign program in DNASTAR.

Figure 9 is an alignment of the amino acid sequence of HSP90 protein of GLRaV-2 with respect to other closteroviruses, BYV, BYSV, and CTV. The most conserved motifs (I to II) are indicated with the highlighted lines and marked with respective letters.

Figure 10 is an alignment of the nucleotide sequence of 3'-terminal untranslated region of GLRaV-2 with respect to the closteroviruses BYV (Agranovsky et al., "Beet Yellows Closterovirus: Complete Genome Structure and Identification of a Papain-like Thiol Protease," Virology 198:311-24 (1994), which is hereby incorporated by reference), BYSV (Karasev et al., Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses," Virology 221:199-207 (1996), which is hereby incorporated by reference), and CTV (Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," Virology 208:511-20 (1995), which is hereby incorporated by reference). The consensus sequences are shown, and the distance to the 3'-end is indicated. A complementary region capable of forming a "hair-pin" structure is underlined.

Figures 11A and 11B are genetic maps of the transformation vectors

pGA482GG/EPT8CP-GLRaV-2 and pGA482G/EPT8CP-GLRaV-2, respectively. As shown in Figures 11A and 11B, the plant expression cassette (EPT8CP-GLRaV-2), which consists of a double cauliflower mosaic virus (CaMV) 35S-enhancer, a CaMV 35S-promoter, an alfalfa mosaic virus (ALMV) RNA4 5' leader sequence, a coat protein gene of GLRaV-2 (CP-GLRaV-2), and a CaMV 35S 3' untranslated region as a terminator, was cloned into the transformation vector by EcoR I restriction site. The CP of GLRaV-2 was cloned into the plant expression vector by Nco I restriction site.

Figure 12 is a PCR analysis of DNA molecules extracted from the leaves of putative transgenic plants using both the CP gene of GLRaV-2 and NPT II gene specific primers. An ethidium bromide-stained gel shows a 720 bp amplified DNA fragment for NPT II gene, and a 653 bp DNA fragment for the entire coding sequence of the CP gene. Lane 1,  $\Phi$ 174 / Hae III DNA Marker; lanes 2-6, transgenic plants from different lines; lane 7, the cp gene of GLRaV-2 of positive control; and lane 8, NPT II gene of positive control.

Figure 13 is a comparison of resistant (right side 3 plants) and susceptible (left side 3 plants) transgenic *Nicotiana benthamiana* plants. Plants are shown 48 days after inoculation with GLRaV-2.

Figure 14 is a northern blot analysis of transgenic *Nicotiana benthamiana* plants. An aliquot of 10 g of total RNA extracted from putative transgenic plants was denatured and loaded onto 1% agarose gel containing formaldehyde. The separated RNAs were transferred to Gene Screen Plus membrane and hybridized with a  $^{32}$ P-labeled DNA probe containing the 3' one third CP gene sequence. Lanes 1, 3, and 4 represent nontransformed control plants without RNA expression. The remaining lanes represent transgenic plants from different lines: lanes 2, 14-17, and 22-27 represent plants with high RNA expression level which are susceptible to GLRaV-2; all other lanes represent plants with undetectable or low RNA expression level which are resistant to GLRaV-2.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to isolated DNA molecules encoding for the proteins or polypeptides of a grapevine leafroll virus (type 2). A substantial portion of the grapevine leafroll virus (type-2) ("GLRaV-2") genome has been sequenced. Within the genome are a plurality of open reading frames ("ORFs") and a 3' untranslated region ("UTR"), each containing DNA molecules in accordance with the present invention. The

DNA molecule which constitutes a substantial portion of the GLRaV-2 genome comprises the nucleotide sequence corresponding to SEQ. ID. No. 1 as follows:

start

TAAACATTGC	GAGAGAACCC	CATTAGCGTC	TCCGGGGTGA	ACTTGGAAG	GTCTGCCGCC	60
GCTCAGGTTA	TTTATTTTCGG	CAGTTTCACG	CAGCCCTTCG	CGTTGTATCC	GCGCCAAGAG	120
AGCGCGATCG	TAAAAACGCA	ACTTCCACCG	GTCAGTGTAG	TGAAGGTGGA	GTGCGTAGCT	180
GCGGAGGTAG	CTCCCGACAG	GGGCGTGGTC	GACAAGAAAC	CTACGTCTGT	TGGCGTTCCC	240
CCGCAGCGCG	GTGTGCTTTC	TTTTCCGACG	GTGGTTCGGA	ACGCGGGCGA	CGTGATAATC	300
ACAGGGGTGG	TGCATGAAGC	CCTGAAGAAA	ATTAAAGACG	GGCTCTTACG	CTTCCGCGTA	360
GGCGGTGACA	TGCGTTTTTC	GAGATTTTTTC	TCATCGAACT	ACGGCTGCAG	ATTCGTCGCG	420
AGCGTGCGTA	CGAACACTAC	AGTTTGGCTA	AATTGCACGA	AAGCGAGTGG	TGAGAAATTC	480
TCACTCGCCG	CCGCGTGACAC	GGCGGATTAC	GTGGCGATGC	TGCGTTATGT	GTGTGGCGGG	540
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CATATGAGGT	ATTTGTGCGC	CTTTTACTGT	CGCCCGTTTA	GAGAGTCGGA	TTATGCCCTC	660
GGAATGTGGC	CTACGGTGGC	GCGTCTCAGG	GCATGCGTTG	AGAAGAACTT	CGGTGTCGAA	720
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GATCCGACAT	CTTTAACCTC	CGTAATAACG	GTGAAGATTA	GCGGTCTTCC	AGGTGGTCTT	900
CCTAAAAATA	TAGCGTTTGG	TGCCTTCCTG	TGCGATATAC	GTTACGTCGA	ACCGGTAGAC	960
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001123:3412360

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 TGGAAACGCT CAACGAATTG TGCGCCGAAG ATGATGTTCA CGTCGACAAA GTAAATGAGA 15240  
 ATGGGGACGA AAATCACGAC CTCGAACTCC AAGAGGAATG TTAACATTG GTTAAGTTTA 15300  
 ACGAAAATGA TTAGTAAATA ATAAATCGAA CGTGGGTGTA TCTACCTGAC GTATCAACTT 15360  
 AAGCTGTTAC TGAGTAATTA AACCAACAAG TGTGGTGTA ATGTGTATGT TGATGTAGAG 15420  
 AAAAATCCGT TTGTAGAACG GTGTTTTTCT CTTCTTTATT TTTAAAAAAA AAATAAAAAA 15480  
 AAAAAAAAAA AAGCGGCCGC 15500

Another DNA molecule of the present invention (GLRaV-2 ORF1a) includes nucleotides 4-7923 of SEQ. ID. No. 1 and is believed to code for a large, grapevine leafroll virus polyprotein containing the conserved domains characteristic of two papain-like proteases, a methyltransferase, and a helicase. This DNA molecule comprises the nucleotide sequence corresponding to SEQ. ID. No. 2 as follows:

ACATTGCGAG AGAACCCCAT TAGCGTCTCC GGGGTGAACT TGGGAAGGTC TGCCGCCGCT 60  
 CAGGTTATTT ATTTCCGCAG TTTCACGCAG CCCTTCGCGT TGTATCCGCG CCAAGAGAGC 120  
 GCGATCGTAA AAACGCAACT TCCACCGGTC AGTGTAGTGA AGGTGGAGTG CGTAGCTGCG 180  
 GAGGTAGCTC CCGACAGGGG CGTGGTCGAC AAGAAACCTA CGTCTGTTGG CGTTCCCCCG 240  
 CAGCGCGGTG TGCTTTCTTT TCCGACGGTG GTTCGGAACC GCGGCGACGT GATAATCACA 300  
 GGGGTGGTGC ATGAAGCCCT GAAGAAAATT AAAGACGGGC TCTTACGCTT CCGCGTAGGC 360  
 GGTGACATGC GTTTTTTCGAG ATTTTCTCA TCGAACTACG GCTGCAGATT CGTCGCGAGC 420  
 GTGCGTACGA AACTACAGT TTGGCTAAAT TGCACGAAAG CGAGTGGTGA GAAATTCTCA 480

CTCGCCGCCG	CGTGCACGGC	GGATTACGTG	GCGATGCTGC	GTTATGTGTG	TGGCGGGAAA	540
TTTCCACTCG	TCCTCATGAG	TAGAGTTATT	TACCCGGATG	GGCGCTGTTA	CTTGGCCCAT	600
ATGAGGTATT	TGTGCGCCTT	TTACTGTCGC	CCGTTTAGAG	AGTCGGATTA	TGCCCTCGGA	660
ATGTGGCCTA	CGGTGGCGCG	TCTCAGGGCA	TGCGTTGAGA	AGAACTTCGG	TGTCGAAGCT	720
TGTGGCATAG	CTCTTCGTGG	CTATTACACC	TCTCGCAATG	TTTATCACTG	TGATTATGAC	780
TCTGCTTATG	TAAAATATTT	TAGAAACCTT	TCCGGCCGCA	TTGGCGGTGG	TTCGTTTCGAT	840
CCGACATCTT	TAACCTCCGT	AATAACGGTG	AAGATTAGCG	GTCTTCCAGG	TGGTCTTCCT	900
AAAAATATAG	CGTTTGGTGC	CTTCCTGTGC	GATATACGTT	AGTTCGAACC	GGTAGACTCG	960
GGCGGCATTC	AATCGAGCGT	TAAGACGAAA	CGTGAAGATG	CGCACCGAAC	CGTAGAGGAA	1020
CGGGCGGCCG	GCGGATCCGT	CGAGCAACCG	CGACAAAAGA	GGATAGATGA	GAAAGGTTGC	1080
GGCAGAGTTC	CTAGTGGAGG	TTTTTCGCAT	CTCCTGGTCG	GCAACCTTAA	CGAAGTTAGG	1140
AGGAAGGTAG	CTGCCGGACT	TCTACGCTTT	CGCGTTGGCG	GTGATATGGA	TTTTCATCGC	1200
<del>TGGTTCTCCA</del>	<del>CGGAAGGGGG</del>	<del>CCACCGCTTG</del>	<del>CTGGTGTGGC</del>	<del>GCGGCTCGAG</del>	<del>CGGGAGCGTG</del>	<del>1260</del>
TGCCTTGAAC	TTTACTCACC	ATCTAAAAAC	TTTTTGCGTT	ACGATGTCTT	GCCCTGTTCT	1320
GGAGACTATG	CAGCGATGTT	TTCTTTCGCG	GCGGGCGGCC	GTTTCCCTTT	AGTTTTGATG	1380
ACTAGAATTA	GATACCCGAA	CGGGTTTTGT	TACTTGGCTC	ACTGCCGGTA	CGCGTGCGCG	1440
TTTCTCTTAA	GGGGTTTTGA	TCCGAAGCGT	TTCGACATCG	GTGCTTTCCC	CACCGCGGCC	1500
AAGCTCAGAA	ACCGTATGGT	TTCGGAGCTT	GGTGAAAGAA	GTTTAGGTTT	GAACCTGTAC	1560
GGCGCATATA	CGTCACGCGG	CGTCTTTCAC	TGCGATTATG	ACGCTAAGTT	TATAAAGGAT	1620
TTGCGTCTTA	TGTCAGCAGT	TATAGCTGGA	AAGGACGGGG	TGGAAGAGGT	GGTACCTTCT	1680
GACATAACTC	CTGCCATGAA	GCAGAAAACG	ATCGAAGCCG	TGTATGATAG	ATTATATGGC	1740
GGCACTGACT	CGTTGCTGAA	ACTGAGCATC	GAGAAAGACT	TAATCGATTT	CAAAAATGAC	1800
GTGCAGAGTT	TGAAGAAAGA	TCGGCCGATT	GTCAAAGTGC	CCTTTTACAT	GTCGGAAGCA	1860
ACACAGAATT	CGCTGACGCG	TTTCTACCCT	CAGTTCGAAC	TTAAGTTTTC	GCACTCCTCG	1920
CATTGAGATC	ATCCCGCCGC	CGCCGCTTCT	AGACTGCTGG	AAAATGAAAC	GTTAGTGCGC	1980
TTATGTGGTA	ATAGCGTTTC	AGATATTGGA	GGTTGTCCTC	TTTTCCATTT	GCATTCCAAG	2040
ACGCAAAGAC	GGGTTCACGT	ATGTAGGCCT	GTGTTGGATG	GCAAGGATGC	GCAGCGTCGC	2100
GTGGTGCGTG	ATTTGCAGTA	TTCCAACGTG	CGTTTGGGAG	ACGATGATAA	AATTTTGGAA	2160
GGGCCACGCA	ATATCGACAT	TTGCCACTAT	CCTCTGGGCG	CGTGTGACCA	CGAAAGTAGT	2220
GCTATGATGA	TGGTGCAGGT	GTATGACGCG	TCCCTTTATG	AGATATGTGG	CGCCATGATC	2280

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AAGAAGAAAA	GCCGCATAAC	GTACTTAACC	ATGGTCACGC	CCGGCGAGTT	TCTTGACGGA	2340
CGCGAATGCG	TCTACATGGA	GTCGTTAGAC	TGTGAGATTG	AAGTTGATGT	GCACGCGGAC	2400
GTCGTAATGT	ACAAATTCGG	TAGTTCTTGC	TATTGCGACA	AGCTTTCAAT	CATCAAGGAC	2460
ATCATGACCA	CTCCGTACTT	GACACTAGGT	GGTTTTCTAT	TCAGCGTGGA	GATGTATGAG	2520
GTGCGTATGG	GCGTGAATTA	CTTCAAGATT	ACGAAGTCCG	AAGTATCGCC	TAGCATTAGC	2580
TGCACCAAGC	TCCTGAGATA	CCGAAGAGCT	AATAGTGACG	TGGTTAAAGT	TAAACTTCCA	2640
CGTTTCGATA	AGAAACGTCG	CATGTGTCTG	CCTGGGTATG	ACACCATATA	CCTAGATTCTG	2700
AAGTTTGTGA	GTCGCGTTTT	CGATTATGTC	GTGTGTAATT	GCTCTGCCGT	GAAGTCAAAA	2760
ACTTTCGAGT	GGGTGTGGAG	TTTCATTAAG	TCTAGTAAGT	CGAGGGTGAT	TATTAGCGGT	2820
AAAATAATTC	ACAAGGATGT	GAATTTGGAC	CTCAAGTACG	TCGAGAGTTT	CGCCGCGGTT	2880
ATGTTGGCCT	CTGGCGTGCG	CAGTAGACTA	GCGTCCGAGT	ACCTTGCTAA	GAACCTTAGT	2940
CATTTTTTCG	GAGATTGCTC	CTTTATTGAA	GCCACGTCTT	TCGTGTTGCG	TGAGAAAAATC	3000
AGAAACATGA	CTCTGAATTT	TAACGAAAGA	CTTTTACAGT	TAGTGAAGCG	CGTTGCCTTT	3060
GCGACCTTGG	ACGTGAGTTT	TCTAGATTTA	GATTCAACTC	TTGAATCAAT	AACTGATTTT	3120
GCCGAGTGTA	AGGTAGCGAT	TGAACTCGAC	GAGTTGGGTT	GCTTGAGAGC	GGAGGCCGAG	3180
AATGAAAAAA	TCAGGAATCT	GGCGGGAGAT	TCGATTGCGG	CTAAACTCGC	GAGCGAGATA	3240
GTGGTCGATA	TTGACTCTAA	GCCTTCACCG	AAGCAGGTGG	GTAATTCGTC	ATCCGAAAAC	3300
GCCGATAAGC	GGGAAGTTCA	GAGGCCCGGT	TTGCGTGGTG	GTTCTAGAAA	CGGGGTTGTT	3360
GGGGAGTTCC	TTCACTTCGT	CGTGGATTCT	GCCTTGCGTC	TTTTCAAATA	CGCGACGGAT	3420
CAACAACGGA	TCAAGTCTTA	CGTGCGTTTC	TTGGACTCGG	CGGTCTCATT	CTTGATTAC	3480
AACTACGATA	ATCTATCGTT	TATACTGCGA	GTGCTTTCGG	AAGGTTATTC	GTGTATGTTT	3540
GCGTTTTTGG	CGATTCGCG	GCACTTATCT	AGTCGTGTCC	GTAGCGCGGT	GTGTGCTGTG	3600
AAAGAAGTTG	CTACCTCATG	CGCGAACGCG	AGCGTTTCTA	AAGCCAAGGT	TATGATTACC	3660
TTCGCAGCGG	CCGTGTGTGC	TATGATGTTT	AATAGCTGCG	GTTTTTCAGG	CGACGGTCGG	3720
GAGTATAAAT	CGTATATACA	TCGTTACACG	CAAGTATTGT	TTGACACTAT	CTTTTTTGAG	3780
GACAGCAGTT	ACCTACCCAT	AGAAGTTCTG	AGTTCGGCGA	TATGCGGTGC	TATCGTCACA	3840
CTTTTCTCCT	CGGGCTCGTC	CATAAGTTTA	AACGCCTTCT	TACTTCAAAT	TACCAAAGGA	3900
TTCTCCCTAG	AGGTTGTGCT	CCGGAATGTT	GTGCGAGTCA	CGCATGGTTT	GAGCACCACA	3960
GCGACCGACG	GCGTCATACG	TGGGGTTTTT	TCCCAAATTG	TGTCTCACTT	ACTTGTTGGA	4020
AATACGGGTA	ATGTGGCTTA	CCAGTCAGCT	TTCATTGCCG	GGGTGGTGCC	TCTTTTAGTT	4080
AAAAAGTGTG	TGAGCTTAAT	CTTCATCTTG	CGTGAAGATA	CTTATTCGGG	TTTTATTAAG	4140

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CACGGAATCA GTGAATTCTC TTTCCTTAGT AGTATTCTGA AGTTCTTGAA GGGTAAGCTT	4200
GTGGACGAGT TGAAATCGAT TATTCAAGGG GTTTTTGATT CCAACAAGCA CGTGTTTAAA	4260
GAAGCTACTC AGGAAGCGAT TCGTACGACG GTCATGCAAG TGCCTGTGCG TGTAGTGGAT	4320
GCCCTTAAGA GCGCCGCGGG AAAAATTTAT AACAATTTTA CTAGTCGACG TACCTTTGGT	4380
AAGGATGAAG GCTCCTCTAG CGACGGCGCA TGTGAAGAGT ATTTCTCATG CGACGAAGGT	4440
GAAGGTCCGG GTCTGAAAGG GGGTTCCAGC TATGGCTTCT CAATTTTAGC GTTCTTTTCA	4500
CGCATTATGT GGGGAGCTCG TCGGCTTATT GTTAAGGTGA AGCATGAGTG TTTTGGGAAA	4560
CTTTTTGAAT TTCTATCGCT CAAGCTTCAC GAATTCAGGA CTCGCGTTTT TGGGAAGAAT	4620
AGAACGGACG TGGGAGTTTA CGATTTTTTG CCCACGGGCA TCGTGGAAC GCTCTCATCG	4680
ATAGAAGAGT GCGACCAAAT TGAAGAACTT CTCGGCGACG ACCTGAAAGG TGACAAGGAT	4740
GCTTCGTTGA CCGATATGAA TTACTTTGAG TTCTCAGAAG ACTTCTTAGC CTCTATCGAG	4800
GAGCCGCCTT TCGCTGGATT GCGAGGAGGT AGCAAGAACA TCGCGATTTT GGCGATTTTG	4860
GAATACGCGC ATAATTTGTT TCGCATTGTC GCAAGCAAGT GTTCGAAACG ACCTTTATTT	4920
CTTGCTTTTCG CCGAACTCTC AAGCGCCCTT ATCGAGAAAT TTAAGGAGGT TTTCCCTCGT	4980
AAGAGCCAGC TCGTCGCTAT CGTGCGCGAG TATACTCAGA GATTCCTCCG AAGTCGCATG	5040
CGTGCGTTGG GTTTGAATAA CGAGTTCGTG GTAAAATCTT TCGCCGATTT GCTACCCGCA	5100
TTAATGAAGC GGAAGGTTTC AGGTTCGTTT TTAGCTAGTG TTTATCGCCC ACTTAGAGGT	5160
TTCTCATATA TGTGTGTTTC AGCGGAGCGA CGTGAAAAGT TTTTGTCTCT CGTGTGTTTA	5220
ATCGGGTTAA GTCTCCCTTT CTTCGTGCGC ATCGTAGGAG CGAAAGCGTG CGAAGAATCT	5280
GTGTCCCTCAG CGCGTCGCTT TTATGAGCGT ATTAAAATTT TTCTAAGGCA GAAGTATGTC	5340
TCTCTTTCTA ATTTCTTTTG TCACTTGTTT AGCTCTGACG TTGATGACAG TTCCGCATCT	5400
GCAGGGTTGA AAGGTGGTGC GTCGCGAATG ACGCTCTTCC ACCTTCTGGT TCGCCTTGCT	5460
AGTGCCCTCC TATCGTTAGG GTGGGAAGGG TTAAAGCTAC TCTTATCGCA CCACAACCTG	5520
TTATTTTTGT GTTTTGCAAT GGTGACGAT GTGAACGTCC TTATCAAAGT TCTTGGGGGT	5580
CTTCTTTCTT TTGTGCAACC AATCTTTTCC TTGTTTGCGG CGATGCTTCT ACAACCGGAC	5640
AGGTTTGTGG AGTATTCCGA GAACTTGTT ACAGCGTTTG AATTTTCTT AAAATGTTTG	5700
CCTCGCGCGC CTGCACTACT CAAAGGGTTT TTTGAGTGCG TGGCGAACAG CACTGTGTCA	5760
AAAACCGTTC GAAGACTTCT TCGCTGTTTC GTGAAGATGC TCAAACCTCG AAAAGGGCGA	5820
GGGTTGCGTG CGGATGGTAG GGGTCTCCAT CGGCAGAAAG CCGTACCCGT CATACCTTCT	5880
AATCGGGTCG TGACCGACGG GGTGAAAGA CTTTCGGTAA AGATGCAAGG AGTTGAAGCG	5940

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TTGCGTACCG AATTGAGAAT CTTAGAAGAT TTAGATTCTG CCGTGATCGA AAAACTCAAT	6000
AGACGCAGAA ATCGTGACAC TAATGACGAC GAATTTACGC GCCCTGCTCA TGAGCAGATG	6060
CAAGAAGTCA CCACTTTCTG TTCGAAAGCC AACTCTGCTG GTTTGGCCCT GGAAAGGGCA	6120
GTGCTTGTGG AAGACGCTAT AAAGTCGGAG AAACCTTCTA AGACGGTTAA TGAGATGGTG	6180
AGGAAAGGGA GTACCACCAG CGAAGAAGTG GCCGTCGCTT TGTCGGACGA TGAAGCCGTG	6240
GAAGAAATCT CTGTTGCTGA CGAGCGAGAC GATTCGCCTA AGACAGTCAG GATAAGCGAA	6300
TACCTAAATA GGTAAACTC AAGCTTCGAA TTCCCGAAGC CTATTGTTGT GGACGACAAC	6360
AAGGATACCG GGGGTCTAAC GAACGCCGTG AGGGAGTTTT ATTATATGCA AGAACTTGCT	6420
CTTTTCGAAA TCCACAGCAA ACTGTGCACC TACTACGATC AACTGCGCAT AGTCAACTTC	6480
GATCGTTCCG TAGCACCATG CAGCGAAGAT GCTCAGCTGT ACGTACGGAA GAACGGCTCA	6540
ACGATAGTGC AGGGTAAAGA GGTACGTTTG CACATTAAGG ATTTCCACGA TCACGATTTT	6600
CTGTTTGACG GAAAAATTC TATTAACAAG CGGCGGCGAG GCGGAAATGT TTTATATCAC	6660
GACAACCTCG CGTTCTTGGC GAGTAATTTG TTCTTAGCCG GCTACCCCTT TTCAAGGAGC	6720
TTCGTCTTCA CGAATTCGTC GGTCGATATT CTCCTCTACG AAGCTCCACC CGGAGGTGGT	6780
AAGACGACGA CGCTGATTGA CTCGTTCTTG AAGGTCTTCA AGAAAGGTGA GGTTCACACC	6840
ATGATCTTAA CCGCCAACAA AAGTTCGCAG GTTGAGATCC TAAAGAAAGT GGAGAAGGAA	6900
GTGTCTAACA TTGAATGCCA GAAACGTAAA GACAAAAGAT CTCCGAAAAA GAGCATTTAC	6960
ACCATCGACG CTTATTTAAT GCATCACCGT GGTGTGATG CAGACGTTCT TTTCATCGAT	7020
GAGTGTTCAT TGGTTCATGC GGGTAGCGTA CTAGCTTGCA TTGAGTTCAC GAGGTGTCAT	7080
AAAGTAATGA TCTTCGGGGA TAGCCGGCAG ATTCACTACA TTGAAAGGAA CGAATTGGAC	7140
AAGTGTTTGT ATGGGGATCT CGACAGGTTC GTGGACCTGC AGTGTCGGGT TTATGGTAAT	7200
ATTTCTGATC GTTGTCTCTG GGATGTGTGC GCTTGTGTTA GCACAGTGTA TGGCAACCTA	7260
ATCGCCACCG TGAAGGGTGA AAGCGAAGGT AAGAGCAGCA TGCGCATTAA CGAAATTAAT	7320
TCAGTCGACG ATTTAGTCCC CGACGTGGGT TCCACGTTTC TGTGTATGCT TCAGTCGGAG	7380
AAGTTGGAAT TCAGCAAGCA CTTTATTCGC AAGGGTTTGA CTAAACTTAA CGTTCTAACG	7440
GTGCATGAGG CGCAAGGTGA GACGTATGCG CGTGTGAACC TTGTGCGACT TAAGTTTCAG	7500
GAGGATGAAC CCTTTAAATC TATCAGGCAC ATAACCGTCG CTCTTTCTCG TCACACCGAC	7560
AGCTTAACTT ATAACGTCTT AGCTGCTCGT CGAGGTGACG CCACTTGCGA TGCCATCCAG	7620
AAGGCTGCGG AATTGGTGAA CAAGTTTCGC GTTTTTCTTA CATCTTTTGG TGGTAGTGTT	7680
ATCAATCTCA ACGTGAAGAA GGACGTGGAA GATAACAGTA GGTGCAAGGC TTCGTCGGCA	7740
CCATTGAGCG TAATCAACGA CTTTTTGAAC GAAGTTAATC CCGGTACTGC GGTGATTGAT	7800

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TTTGGTGATT TGTCCGCGGA CTTCAGTACT GGGCCTTTTG AGTGCGGTGC CAGCGGTATT 7860  
GTGGTGCGGG ACAACATCTC CTCCAGCAAC ATCACTGATC ACGATAAGCA GCGTGTTTAG 7920

The large polypeptide (papain-like proteases, methyltransferase, and helicase) has an amino acid sequence corresponding to SEQ. ID. No. 3 as follows:

Thr	Leu	Arg	Glu	Asn	Pro	Ile	Ser	Val	Ser	Gly	Val	Asn	Leu	Gly	Arg	1	5	10	15
Ser	Ala	Ala	Ala	Gln	Val	Ile	Tyr	Phe	Gly	Ser	Phe	Thr	Gln	Pro	Phe	20	25	30	
Ala	Leu	Tyr	Pro	Arg	Gln	Glu	Ser	Ala	Ile	Val	Lys	Thr	Gln	Leu	Pro	35	40	45	
Pro	Val	Ser	Val	Val	Lys	Val	Glu	Cys	Val	Ala	Ala	Glu	Val	Ala	Pro	50	55	60	
Asp	Arg	Gly	Val	Val	Asp	Lys	Lys	Pro	Thr	Ser	Val	Gly	Val	Pro	Pro	65	70	75	80
Gln	Arg	Gly	Val	Leu	Ser	Phe	Pro	Thr	Val	Val	Arg	Asn	Arg	Gly	Asp	85	90	95	
Val	Ile	Ile	Thr	Gly	Val	Val	His	Glu	Ala	Leu	Lys	Lys	Ile	Lys	Asp	100	105	110	
Gly	Leu	Leu	Arg	Phe	Arg	Val	Gly	Gly	Asp	Met	Arg	Phe	Ser	Arg	Phe	115	120	125	
Phe	Ser	Ser	Asn	Tyr	Gly	Cys	Arg	Phe	Val	Ala	Ser	Val	Arg	Thr	Asn	130	135	140	
Thr	Thr	Val	Trp	Leu	Asn	Cys	Thr	Lys	Ala	Ser	Gly	Glu	Lys	Phe	Ser	145	150	155	160
Leu	Ala	Ala	Ala	Cys	Thr	Ala	Asp	Tyr	Val	Ala	Met	Leu	Arg	Tyr	Val	165	170	175	
Cys	Gly	Gly	Lys	Phe	Pro	Leu	Val	Leu	Met	Ser	Arg	Val	Ile	Tyr	Pro	180	185	190	
Asp	Gly	Arg	Cys	Tyr	Leu	Ala	His	Met	Arg	Tyr	Leu	Cys	Ala	Phe	Tyr	195	200	205	
Cys	Arg	Pro	Phe	Arg	Glu	Ser	Asp	Tyr	Ala	Leu	Gly	Met	Trp	Pro	Thr	210	215	220	
Val	Ala	Arg	Leu	Arg	Ala	Cys	Val	Glu	Lys	Asn	Phe	Gly	Val	Glu	Ala	225	230	235	240
Cys	Gly	Ile	Ala	Leu	Arg	Gly	Tyr	Tyr	Thr	Ser	Arg	Asn	Val	Tyr	His	245	250	255	
Cys	Asp	Tyr	Asp	Ser	Ala	Tyr	Val	Lys	Tyr	Phe	Arg	Asn	Leu	Ser	Gly	260	265	270	

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Arg	Ile	Gly	Gly	Ser	Phe	Asp	Pro	Thr	Ser	Leu	Thr	Ser	Val	Ile	
	275					280					285				
Thr	Val	Lys	Ile	Ser	Gly	Leu	Pro	Gly	Gly	Leu	Pro	Lys	Asn	Ile	Ala
	290					295					300				
Phe	Gly	Ala	Phe	Leu	Cys	Asp	Ile	Arg	Tyr	Val	Glu	Pro	Val	Asp	Ser
305					310					315					320
Gly	Gly	Ile	Gln	Ser	Ser	Val	Lys	Thr	Lys	Arg	Glu	Asp	Ala	His	Arg
				325					330					335	
Thr	Val	Glu	Glu	Arg	Ala	Ala	Gly	Gly	Ser	Val	Glu	Gln	Pro	Arg	Gln
			340					345					350		
Lys	Arg	Ile	Asp	Glu	Lys	Gly	Cys	Gly	Arg	Val	Pro	Ser	Gly	Gly	Phe
		355					360					365			
Ser	His	Leu	Leu	Val	Gly	Asn	Leu	Asn	Glu	Val	Arg	Arg	Lys	Val	Ala
	370					375					380				
Ala	Gly	Leu	Leu	Arg	Phe	Arg	Val	Gly	Gly	Asp	Met	Asp	Phe	His	Arg
385					390					395					400
Ser	Phe	Ser	Thr	Gln	Ala	Gly	His	Arg	Leu	Leu	Val	Trp	Arg	Arg	Ser
				405					410					415	
Ser	Arg	Ser	Val	Cys	Leu	Glu	Leu	Tyr	Ser	Pro	Ser	Lys	Asn	Phe	Leu
			420					425					430		
Arg	Tyr	Asp	Val	Leu	Pro	Cys	Ser	Gly	Asp	Tyr	Ala	Ala	Met	Phe	Ser
		435					440					445			
Phe	Ala	Ala	Gly	Gly	Arg	Phe	Pro	Leu	Val	Leu	Met	Thr	Arg	Ile	Arg
	450					455					460				
Tyr	Pro	Asn	Gly	Phe	Cys	Tyr	Leu	Ala	His	Cys	Arg	Tyr	Ala	Cys	Ala
465					470					475					480
Phe	Leu	Leu	Arg	Gly	Phe	Asp	Pro	Lys	Arg	Phe	Asp	Ile	Gly	Ala	Phe
				485					490					495	
Pro	Thr	Ala	Ala	Lys	Leu	Arg	Asn	Arg	Met	Val	Ser	Glu	Leu	Gly	Glu
			500					505					510		
Arg	Ser	Leu	Gly	Leu	Asn	Leu	Tyr	Gly	Ala	Tyr	Thr	Ser	Arg	Gly	Val
		515					520					525			
Phe	His	Cys	Asp	Tyr	Asp	Ala	Lys	Phe	Ile	Lys	Asp	Leu	Arg	Leu	Met
	530					535					540				
Ser	Ala	Val	Ile	Ala	Gly	Lys	Asp	Gly	Val	Glu	Glu	Val	Val	Pro	Ser
545					550					555					560
Asp	Ile	Thr	Pro	Ala	Met	Lys	Gln	Lys	Thr	Ile	Glu	Ala	Val	Tyr	Asp
				565					570					575	
Arg	Leu	Tyr	Gly	Gly	Thr	Asp	Ser	Leu	Leu	Lys	Leu	Ser	Ile	Glu	Lys
			580					585					590		

Asp Leu Ile Asp Phe Lys Asn Asp Val Gln Ser Leu Lys Lys Asp Arg  
595 600 605

Pro Ile Val Lys Val Pro Phe Tyr Met Ser Glu Ala Thr Gln Asn Ser  
610 615 620

Leu Thr Arg Phe Tyr Pro Gln Phe Glu Leu Lys Phe Ser His Ser Ser  
625 630 635 640

His Ser Asp His Pro Ala Ala Ala Ala Ser Arg Leu Leu Glu Asn Glu  
645 650 655

Thr Leu Val Arg Leu Cys Gly Asn Ser Val Ser Asp Ile Gly Gly Cys  
660 665 670

Pro Leu Phe His Leu His Ser Lys Thr Gln Arg Arg Val His Val Cys  
675 680 685

Arg Pro Val Leu Asp Gly Lys Asp Ala Gln Arg Arg Val Val Arg Asp  
690 695 700

Leu Gln Tyr Ser Asn Val Arg Leu Gly Asp Asp Lys Ile Leu Glu  
705 710 715 720

Gly Pro Arg Asn Ile Asp Ile Cys His Tyr Pro Leu Gly Ala Cys Asp  
725 730 735

His Glu Ser Ser Ala Met Met Met Val Gln Val Tyr Asp Ala Ser Leu  
740 745 750

Tyr Glu Ile Cys Gly Ala Met Ile Lys Lys Lys Ser Arg Ile Thr Tyr  
755 760 765

Leu Thr Met Val Thr Pro Gly Glu Phe Leu Asp Gly Arg Glu Cys Val  
770 775 780

Tyr Met Glu Ser Leu Asp Cys Glu Ile Glu Val Asp Val His Ala Asp  
785 790 795 800

Val Val Met Tyr Lys Phe Gly Ser Ser Cys Tyr Ser His Lys Leu Ser  
805 810 815

Ile Ile Lys Asp Ile Met Thr Thr Pro Tyr Leu Thr Leu Gly Gly Phe  
820 825 830

Leu Phe Ser Val Glu Met Tyr Glu Val Arg Met Gly Val Asn Tyr Phe  
835 840 845

Lys Ile Thr Lys Ser Glu Val Ser Pro Ser Ile Ser Cys Thr Lys Leu  
850 855 860

Leu Arg Tyr Arg Arg Ala Asn Ser Asp Val Val Lys Val Lys Leu Pro  
865 870 875 880

Arg Phe Asp Lys Lys Arg Arg Met Cys Leu Pro Gly Tyr Asp Thr Ile  
885 890 895

Tyr Leu Asp Ser Lys Phe Val Ser Arg Val Phe Asp Tyr Val Val Cys  
900 905 910

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Asn	Cys	Ser	Ala	Val	Asn	Ser	Lys	Thr	Phe	Glu	Trp	Val	Trp	Ser	Phe
	915						920					925			
Ile	Lys	Ser	Ser	Lys	Ser	Arg	Val	Ile	Ile	Ser	Gly	Lys	Ile	Ile	His
	930					935					940				
Lys	Asp	Val	Asn	Leu	Asp	Leu	Lys	Tyr	Val	Glu	Ser	Phe	Ala	Ala	Val
945					950					955					960
Met	Leu	Ala	Ser	Gly	Val	Arg	Ser	Arg	Leu	Ala	Ser	Glu	Tyr	Leu	Ala
				965					970					975	
Lys	Asn	Leu	Ser	His	Phe	Ser	Gly	Asp	Cys	Ser	Phe	Ile	Glu	Ala	Thr
			980					985					990		
Ser	Phe	Val	Leu	Arg	Glu	Lys	Ile	Arg	Asn	Met	Thr	Leu	Asn	Phe	Asn
		995					1000					1005			
Glu	Arg	Leu	Leu	Gln	Leu	Val	Lys	Arg	Val	Ala	Phe	Ala	Thr	Leu	Asp
	1010					1015					1020				
Val	Ser	Phe	Leu	Asp	Leu	Asp	Ser	Thr	Leu	Glu	Ser	Ile	Thr	Asp	Phe
1025					1030					1035					1040
Ala	Glu	Cys	Lys	Val	Ala	Ile	Glu	Leu	Asp	Glu	Leu	Gly	Cys	Leu	Arg
			1045						1050					1055	
Ala	Glu	Ala	Glu	Asn	Glu	Lys	Ile	Arg	Asn	Leu	Ala	Gly	Asp	Ser	Ile
			1060					1065					1070		
Ala	Ala	Lys	Leu	Ala	Ser	Glu	Ile	Val	Val	Asp	Ile	Asp	Ser	Lys	Pro
		1075					1080					1085			
Ser	Pro	Lys	Gln	Val	Gly	Asn	Ser	Ser	Ser	Glu	Asn	Ala	Asp	Lys	Arg
	1090					1095					1100				
Glu	Val	Gln	Arg	Pro	Gly	Leu	Arg	Gly	Gly	Ser	Arg	Asn	Gly	Val	Val
1105					1110					1115					1120
Gly	Glu	Phe	Leu	His	Phe	Val	Val	Asp	Ser	Ala	Leu	Arg	Leu	Phe	Lys
				1125					1130					1135	
Tyr	Ala	Thr	Asp	Gln	Gln	Arg	Ile	Lys	Ser	Tyr	Val	Arg	Phe	Leu	Asp
			1140					1145					1150		
Ser	Ala	Val	Ser	Phe	Leu	Asp	Tyr	Asn	Tyr	Asp	Asn	Leu	Ser	Phe	Ile
		1155					1160					1165			
Leu	Arg	Val	Leu	Ser	Glu	Gly	Tyr	Ser	Cys	Met	Phe	Ala	Phe	Leu	Ala
	1170					1175					1180				
Asn	Arg	Gly	Asp	Leu	Ser	Ser	Arg	Val	Arg	Ser	Ala	Val	Cys	Ala	Val
1185					1190					1195					1200
Lys	Glu	Val	Ala	Thr	Ser	Cys	Ala	Asn	Ala	Ser	Val	Ser	Lys	Ala	Lys
				1205					1210					1215	
Val	Met	Ile	Thr	Phe	Ala	Ala	Ala	Val	Cys	Ala	Met	Met	Phe	Asn	Ser
			1220					1225					1230		

Cys Gly Phe Ser Gly Asp Gly Arg Glu Tyr Lys Ser Tyr Ile His Arg  
1235 1240 1245

Tyr Thr Gln Val Leu Phe Asp Thr Ile Phe Phe Glu Asp Ser Ser Tyr  
1250 1255 1260

Leu Pro Ile Glu Val Leu Ser Ser Ala Ile Cys Gly Ala Ile Val Thr  
1265 1270 1275 1280

Leu Phe Ser Ser Gly Ser Ser Ile Ser Leu Asn Ala Phe Leu Leu Gln  
1285 1290 1295

Ile Thr Lys Gly Phe Ser Leu Glu Val Val Val Arg Asn Val Val Arg  
1300 1305 1310

Val Thr His Gly Leu Ser Thr Thr Ala Thr Asp Gly Val Ile Arg Gly  
1315 1320 1325

Val Phe Ser Gln Ile Val Ser His Leu Leu Val Gly Asn Thr Gly Asn  
1330 1335 1340

Val Ala Tyr Gln Ser Ala Phe Ile Ala Gly Val Val Pro Leu Leu Val  
1345 1350 1355 1360

Lys Lys Cys Val Ser Leu Ile Phe Ile Leu Arg Glu Asp Thr Tyr Ser  
1365 1370 1375

Gly Phe Ile Lys His Gly Ile Ser Glu Phe Ser Phe Leu Ser Ser Ile  
1380 1385 1390

Leu Lys Phe Leu Lys Gly Lys Leu Val Asp Glu Leu Lys Ser Ile Ile  
1395 1400 1405

Gln Gly Val Phe Asp Ser Asn Lys His Val Phe Lys Glu Ala Thr Gln  
1410 1415 1420

Glu Ala Ile Arg Thr Thr Val Met Gln Val Pro Val Ala Val Val Asp  
1425 1430 1435 1440

Ala Leu Lys Ser Ala Ala Gly Lys Ile Tyr Asn Asn Phe Thr Ser Arg  
1445 1450 1455

Arg Thr Phe Gly Lys Asp Glu Gly Ser Ser Ser Asp Gly Ala Cys Glu  
1460 1465 1470

Glu Tyr Phe Ser Cys Asp Glu Gly Glu Gly Pro Gly Leu Lys Gly Gly  
1475 1480 1485

Ser Ser Tyr Gly Phe Ser Ile Leu Ala Phe Phe Ser Arg Ile Met Trp  
1490 1495 1500

Gly Ala Arg Arg Leu Ile Val Lys Val Lys His Glu Cys Phe Gly Lys  
1505 1510 1515 1520

Leu Phe Glu Phe Leu Ser Leu Lys Leu His Glu Phe Arg Thr Arg Val  
1525 1530 1535

Phe Gly Lys Asn Arg Thr Asp Val Gly Val Tyr Asp Phe Leu Pro Thr  
1540 1545 1550

00720 5547560

Gly Ile Val Glu Thr Leu Ser Ser Ile Glu Glu Cys Asp Gln Ile Glu  
1555 1560 1565

Glu Leu Leu Gly Asp Asp Leu Lys Gly Asp Lys Asp Ala Ser Leu Thr  
1570 1575 1580

Asp Met Asn Tyr Phe Glu Phe Ser Glu Asp Phe Leu Ala Ser Ile Glu  
1585 1590 1595 1600

Glu Pro Pro Phe Ala Gly Leu Arg Gly Gly Ser Lys Asn Ile Ala Ile  
1605 1610 1615

Leu Ala Ile Leu Glu Tyr Ala His Asn Leu Phe Arg Ile Val Ala Ser  
1620 1625 1630

Lys Cys Ser Lys Arg Pro Leu Phe Leu Ala Phe Ala Glu Leu Ser Ser  
1635 1640 1645

Ala Leu Ile Glu Lys Phe Lys Glu Val Phe Pro Arg Lys Ser Gln Leu  
1650 1655 1660

Val Ala Ile Val Arg Glu Tyr Thr Gln Arg Phe Leu Arg Ser Arg Met  
1665 1670 1675 1680

Arg Ala Leu Gly Leu Asn Asn Glu Phe Val Val Lys Ser Phe Ala Asp  
1685 1690 1695

Leu Leu Pro Ala Leu Met Lys Arg Lys Val Ser Gly Ser Phe Leu Ala  
1700 1705 1710

Ser Val Tyr Arg Pro Leu Arg Gly Phe Ser Tyr Met Cys Val Ser Ala  
1715 1720 1725

Glu Arg Arg Glu Lys Phe Phe Ala Leu Val Cys Leu Ile Gly Leu Ser  
1730 1735 1740

Leu Pro Phe Phe Val Arg Ile Val Gly Ala Lys Ala Cys Glu Glu Leu  
1745 1750 1755 1760

Val Ser Ser Ala Arg Arg Phe Tyr Glu Arg Ile Lys Ile Phe Leu Arg  
1765 1770 1775

Gln Lys Tyr Val Ser Leu Ser Asn Phe Phe Cys His Leu Phe Ser Ser  
1780 1785 1790

Asp Val Asp Asp Ser Ser Ala Ser Ala Gly Leu Lys Gly Gly Ala Ser  
1795 1800 1805

Arg Met Thr Leu Phe His Leu Leu Val Arg Leu Ala Ser Ala Leu Leu  
1810 1815 1820

Ser Leu Gly Trp Glu Gly Leu Lys Leu Leu Leu Ser His His Asn Leu  
1825 1830 1835 1840

Leu Phe Leu Cys Phe Ala Leu Val Asp Asp Val Asn Val Leu Ile Lys  
1845 1850 1855

Val Leu Gly Gly Leu Ser Phe Phe Val Gln Pro Ile Phe Ser Leu Phe  
1860 1865 1870

001101 98011960

Ala	Ala	Met	Leu	Leu	Gln	Pro	Asp	Arg	Phe	Val	Glu	Tyr	Ser	Glu	Lys	
1875						1880						1885				
Leu	Val	Thr	Ala	Phe	Glu	Phe	Phe	Leu	Lys	Cys	Ser	Pro	Arg	Ala	Pro	
1890						1895						1900				
Ala	Leu	Leu	Lys	Gly	Phe	Phe	Glu	Cys	Val	Ala	Asn	Ser	Thr	Val	Ser	
1905						1910						1915			1920	
Lys	Thr	Val	Arg	Arg	Leu	Leu	Arg	Cys	Phe	Val	Lys	Met	Leu	Lys	Leu	
			1925						1930						1935	
Arg	Lys	Gly	Arg	Gly	Leu	Arg	Ala	Asp	Gly	Arg	Gly	Leu	His	Arg	Gln	
			1940						1945						1950	
Lys	Ala	Val	Pro	Val	Ile	Pro	Ser	Asn	Arg	Val	Val	Thr	Asp	Gly	Val	
1955						1960						1965				
Glu	Arg	Leu	Ser	Val	Lys	Met	Gln	Gly	Val	Glu	Ala	Leu	Arg	Thr	Glu	
1970						1975						1980				
Leu	Arg	Ile	Leu	Glu	Asp	Leu	Asp	Ser	Ala	Val	Ile	Glu	Lys	Leu	Asn	
1985						1990						1995			2000	
Arg	Arg	Arg	Asn	Arg	Asp	Thr	Asn	Asp	Asp	Glu	Phe	Thr	Arg	Pro	Ala	
			2005						2010						2015	
His	Glu	Gln	Met	Gln	Glu	Val	Thr	Thr	Phe	Cys	Ser	Lys	Ala	Asn	Ser	
			2020						2025						2030	
Ala	Gly	Leu	Ala	Leu	Glu	Arg	Ala	Val	Leu	Val	Glu	Asp	Ala	Ile	Lys	
2035						2040						2045				
Ser	Glu	Lys	Leu	Ser	Lys	Thr	Val	Asn	Glu	Met	Val	Arg	Lys	Gly	Ser	
2050						2055						2060				
Thr	Thr	Ser	Glu	Glu	Val	Ala	Val	Ala	Leu	Ser	Asp	Asp	Glu	Ala	Val	
2065						2070						2075			2080	
Glu	Glu	Ile	Ser	Val	Ala	Asp	Glu	Arg	Asp	Asp	Ser	Pro	Lys	Thr	Val	
			2085						2090						2095	
Arg	Ile	Ser	Glu	Tyr	Leu	Asn	Arg	Leu	Asn	Ser	Ser	Phe	Glu	Phe	Pro	
			2100						2105						2110	
Lys	Pro	Ile	Val	Val	Asp	Asp	Asn	Lys	Asp	Thr	Gly	Gly	Leu	Thr	Asn	
2115						2120						2125				
Ala	Val	Arg	Glu	Phe	Tyr	Tyr	Met	Gln	Glu	Leu	Ala	Leu	Phe	Glu	Ile	
2130						2135						2140				
His	Ser	Lys	Leu	Cys	Thr	Tyr	Tyr	Asp	Gln	Leu	Arg	Ile	Val	Asn	Phe	
2145						2150						2155			2160	
Asp	Arg	Ser	Val	Ala	Pro	Cys	Ser	Glu	Asp	Ala	Gln	Leu	Tyr	Val	Arg	
			2165						2170						2175	
Lys	Asn	Gly	Ser	Thr	Ile	Val	Gln	Gly	Lys	Glu	Val	Arg	Leu	His	Ile	
			2180						2185						2190	



Lys Asp Phe His Asp His Asp Phe Leu Phe Asp Gly Lys Ile Ser Ile  
2195 2200 2205

Asn Lys Arg Arg Arg Gly Gly Asn Val Leu Tyr His Asp Asn Leu Ala  
2210 2215 2220

Phe Leu Ala Ser Asn Leu Phe Leu Ala Gly Tyr Pro Phe Ser Arg Ser  
2225 2230 2235 2240

Phe Val Phe Thr Asn Ser Ser Val Asp Ile Leu Leu Tyr Glu Ala Pro  
2245 2250 2255

Pro Gly Gly Gly Lys Thr Thr Thr Leu Ile Asp Ser Phe Leu Lys Val  
2260 2265 2270

Phe Lys Lys Gly Glu Val Ser Thr Met Ile Leu Thr Ala Asn Lys Ser  
2275 2280 2285

Ser Gln Val Glu Ile Leu Lys Lys Val Glu Lys Glu Val Ser Asn Ile  
2290 2295 2300

Glu Cys Gln Lys Arg Lys Asp Lys Arg Ser Pro Lys Lys Ser Ile Tyr  
2305 2310 2315 2320

Thr Ile Asp Ala Tyr Leu Met His His Arg Gly Cys Asp Ala Asp Val  
2325 2330 2335

Leu Phe Ile Asp Glu Cys Phe Met Val His Ala Gly Ser Val Leu Ala  
2340 2345 2350

Cys Ile Glu Phe Thr Arg Cys His Lys Val Met Ile Phe Gly Asp Ser  
2355 2360 2365

Arg Gln Ile His Tyr Ile Glu Arg Asn Glu Leu Asp Lys Cys Leu Tyr  
2370 2375 2380

Gly Asp Leu Asp Arg Phe Val Asp Leu Gln Cys Arg Val Tyr Gly Asn  
2385 2390 2395 2400

Ile Ser Tyr Arg Cys Pro Trp Asp Val Cys Ala Trp Leu Ser Thr Val  
2405 2410 2415

Tyr Gly Asn Leu Ile Ala Thr Val Lys Gly Glu Ser Glu Gly Lys Ser  
2420 2425 2430

Ser Met Arg Ile Asn Glu Ile Asn Ser Val Asp Asp Leu Val Pro Asp  
2435 2440 2445

Val Gly Ser Thr Phe Leu Cys Met Leu Gln Ser Glu Lys Leu Glu Ile  
2450 2455 2460

Ser Lys His Phe Ile Arg Lys Gly Leu Thr Lys Leu Asn Val Leu Thr  
2465 2470 2475 2480

Val His Glu Ala Gln Gly Glu Thr Tyr Ala Arg Val Asn Leu Val Arg  
2485 2490 2495

Leu Lys Phe Gln Glu Asp Glu Pro Phe Lys Ser Ile Arg His Ile Thr  
2500 2505 2510

00643436-0440

Val Ala Leu Ser Arg His Thr Asp Ser Leu Thr Tyr Asn Val Leu Ala  
2515 2520 2525

Ala Arg Arg Gly Asp Ala Thr Cys Asp Ala Ile Gln Lys Ala Ala Glu  
2530 2535 2540

Leu Val Asn Lys Phe Arg Val Phe Pro Thr Ser Phe Gly Gly Ser Val  
2545 2550 2555 2560

Ile Asn Leu Asn Val Lys Lys Asp Val Glu Asp Asn Ser Arg Cys Lys  
2565 2570 2575

Ala Ser Ser Ala Pro Leu Ser Val Ile Asn Asp Phe Leu Asn Glu Val  
2580 2585 2590

Asn Pro Gly Thr Ala Val Ile Asp Phe Gly Asp Leu Ser Ala Asp Phe  
2595 2600 2605

Ser Thr Gly Pro Phe Glu Cys Gly Ala Ser Gly Ile Val Val Arg Asp  
2610 2615 2620

Asn Ile Ser Ser Ser Asn Ile Thr Asp His Asp Lys Gln Arg Val  
2625 2630 2635

2636 2637 2638 2639 2640

and has a molecular weight of about 290 to 300 kDa, preferably 294 kDa.

Another such DNA molecule (GLRaV-2 ORF1b) includes nucleotides 7922-9301 of SEQ. ID. No. 1 and codes for a grapevine leafroll virus RNA-dependent RNA polymerase (RdRP). This DNA molecule comprises the nucleotide sequence

5 corresponding to SEQ. ID. No. 4 as follows:

AGCGTAGTTC GGTGCGAGGC GATTCCGCGT AGAAAACCTT CTCTACAAGA AAATTTGTAT 60

TCGTTTGAAG CGCGGAATTA TAACTTCTCG ACTTGCAGACC GTAACACATC TGCTTCAATG 120

TTCGGAGAGG CTATGGCGAT GAACTGTCTT CGTCGTTGCT TCGACCTAGA TGCCTTTTTCG 180

TCCCTGCGTG ATGATGTGAT TAGTATCACA CGTTCAGGCA TCGAACAATG GCTGGAGAAA 240

CGTACTCCTA GTCAGATTAA AGCATTAATG AAGGATGTTG AATCGCCTTT GGAAATTGAC 300

GATGAAATTT GTCGTTTTTAA GTTGATGGTG AAGCGTGACG CTAAGGTGAA GTTAGACTCT 360

TCTTGTTTTAA CTAAACACAG CGCCGCTCAA AATATCATGT TTCATCGCAA GAGCATTAAT 420

GCTATCTTCT CTCCTATCTT TAATGAGGTG AAAAACCGAA TAATGTGCTG TCTTAAGCCT 480

AACATAAAGT TTTTACGGA GATGACTAAC AGGGATTTTG CTTCTGTTGT CAGCAACATG 540

CTTGGTGACG ACGATGTGTA CCATATAGGT GAAGTTGATT TCTCAAAGTA CGACAAGTCT 600

CAAGATGCTT TCGTGAAGGC TTTTGAAGAA GTAATGTATA AGGAACTCGG TGTTGATGAA 660

GAGTTGCTGG CTATCTGGAT GTGCGGCGAG CGGTTATCGA TAGCTAACAC TCTCGATGGT 720

CAGTTGTCCT TCACGATCGA GAATCAAAGG AAGTCGGGAG CTTCGAACAC TTGGATTGGT 780

The RNA-dependent RNA polymerase has an amino acid sequence corresponding to SEQ. ID. No. 5 as follows:

Ser 1	Val	Val	Arg	Ser 5	Gln	Ala	Ile	Pro	Arg 10	Arg	Lys	Pro	Ser	Leu 15	Gln
Glu	Asn	Leu	Tyr 20	Ser	Phe	Glu	Ala	Arg 25	Asn	Tyr	Asn	Phe	Ser 30	Thr	Cys
Asp	Arg	Asn 35	Thr	Ser	Ala	Ser	Met 40	Phe	Gly	Glu	Ala	Met 45	Ala	Met	Asn
Cys	Leu 50	Arg	Arg	Cys	Phe	Asp 55	Leu	Asp	Ala	Phe	Ser 60	Ser	Leu	Arg	Asp
Asp 65	Val	Ile	Ser	Ile	Thr 70	Arg	Ser	Gly	Ile	Glu 75	Gln	Trp	Leu	Glu	Lys 80
Arg	Thr	Pro	Ser	Gln 85	Ile	Lys	Ala	Leu	Met 90	Lys	Asp	Val	Glu	Ser 95	Pro
Leu	Glu	Ile	Asp 100	Asp	Glu	Ile	Cys	Arg 105	Phe	Lys	Leu	Met	Val 110	Lys	Arg
Asp	Ala	Lys 115	Val	Lys	Leu	Asp	Ser 120	Ser	Cys	Leu	Thr	Lys 125	His	Ser	Ala
Ala	Gln 130	Asn	Ile	Met	Phe	His 135	Arg	Lys	Ser	Ile	Asn 140	Ala	Ile	Phe	Ser
Pro 145	Ile	Phe	Asn	Glu	Val 150	Lys	Asn	Arg	Ile	Met 155	Cys	Cys	Leu	Lys	Pro 160
Asn	Ile	Lys	Phe	Phe 165	Thr	Glu	Met	Thr	Asn 170	Arg	Asp	Phe	Ala	Ser 175	Val
Val	Ser	Asn 180	Met	Leu	Gly	Asp	Asp	Asp 185	Val	Tyr	His	Ile	Gly 190	Glu	Val

Asp	Phe	Ser	Lys	Tyr	Asp	Lys	Ser	Gln	Asp	Ala	Phe	Val	Lys	Ala	Phe				
		195					200					205							
Glu	Glu	Val	Met	Tyr	Lys	Glu	Leu	Gly	Val	Asp	Glu	Glu	Leu	Leu	Ala				
	210					215					220								
Ile	Trp	Met	Cys	Gly	Glu	Arg	Leu	Ser	Ile	Ala	Asn	Thr	Leu	Asp	Gly				
225					230					235					240				
Gln	Leu	Ser	Phe	Thr	Ile	Glu	Asn	Gln	Arg	Lys	Ser	Gly	Ala	Ser	Asn				
				245					250					255					
Thr	Trp	Ile	Gly	Asn	Ser	Leu	Val	Thr	Leu	Gly	Ile	Leu	Ser	Leu	Tyr				
			260					265					270						
Tyr	Asp	Val	Arg	Asn	Phe	Glu	Ala	Leu	Tyr	Ile	Ser	Gly	Asp	Asp	Ser				
		275					280					285							
Leu	Ile	Phe	Ser	Arg	Ser	Glu	Ile	Ser	Asn	Tyr	Ala	Asp	Asp	Ile	Cys				
	290					295					300								
Thr	Asp	Met	Gly	Phe	Glu	Thr	Lys	Phe	Met	Ser	Pro	Ser	Val	Pro	Tyr				
305					310					315					320				
<del>Phe</del>	<del>Cys</del>	<del>Ser</del>	<del>Lys</del>	<del>Phe</del>	<del>Val</del>	<del>Val</del>	<del>Met</del>	<del>Cys</del>	<del>Gly</del>	<del>His</del>	<del>Lys</del>	<del>Thr</del>	<del>Phe</del>	<del>Phe</del>	<del>Val</del>				
				325					330					335					
Pro	Asp	Pro	Tyr	Lys	Leu	Phe	Val	Lys	Leu	Gly	Ala	Val	Lys	Glu	Asp				
			340					345					350						
Val	Ser	Met	Asp	Phe	Leu	Phe	Glu	Thr	Phe	Thr	Ser	Phe	Lys	Asp	Leu				
		355					360					365							
Thr	Ser	Asp	Phe	Asn	Asp	Glu	Arg	Leu	Ile	Gln	Lys	Leu	Ala	Glu	Leu				
		370				375					380								
Val	Ala	Leu	Lys	Tyr	Glu	Val	Gln	Thr	Gly	Asn	Thr	Thr	Leu	Ala	Leu				
385					390					395					400				
Ser	Val	Ile	His	Cys	Leu	Arg	Ser	Asn	Phe	Leu	Ser	Phe	Ser	Lys	Leu				
				405					410					415					
Tyr	Pro	Arg	Val	Lys	Gly	Trp	Gln	Val	Phe	Tyr	Thr	Ser	Val	Lys	Lys				
			420				425						430						
Ala	Leu	Leu	Lys	Ser	Gly	Cys	Ser	Leu	Phe	Asp	Ser	Phe	Met	Thr	Pro				
		435					440					445							
Phe	Gly	Gln	Ala	Val	Met	Val	Trp	Asp	Asp	Glu									
	450					455													

and a molecular weight from about 50 to about 54 kDa, preferably about 52 kDa.

Another such DNA molecule (GLRAV-2 ORF2) includes nucleotides 9365-9535 of SEQ. ID. No. 1 and codes for a small, grapevine leafroll virus hydrophobic protein or polypeptide. This DNA molecule comprises the nucleotide sequence

5 corresponding to SEQ. ID. No. 6 as follows:

ATGAATCAGG TTTTGCAGTT TGAATGTTTG TTTCTGCTGA ATCTCGCGGT TTTTGCTGTG 60  
 ACTTTCATTT TCATTCTTCT GGTCTTCCGC GTGATTAAGT CTTTTCGCCA GAAGGGTCAC 120  
 GAAGCACCTG TTCCCGTTGT TCGTGGCGGG GGTTTTTCAA CCGTAGTGTA G 171

The small hydrophobic protein or polypeptide has an amino acid sequence corresponding to SEQ. ID. No. 7 as follows:

Met Asn Gln Val Leu Gln Phe Glu Cys Leu Phe Leu Leu Asn Leu Ala  
 1 5 10 15  
 Val Phe Ala Val Thr Phe Ile Phe Ile Leu Leu Val Phe Arg Val Ile  
 20 25 30  
 Lys Ser Phe Arg Gln Lys Gly His Glu Ala Pro Val Pro Val Val Arg  
 35 40 45  
 Gly Gly Gly Phe Ser Thr Val Val  
 50 55

and a molecular weight from about 5 to about 7 kDa, preferably about 6 kDa.

Another such DNA molecule (GLRaV-2 ORF3) includes nucleotides  
 5 9551-11350 of SEQ. ID. No. 1 and encodes for a grapevine leafroll virus heat shock 70  
 protein. This DNA molecule comprises the nucleotide sequence corresponding to SEQ.  
 ID. No. 8 as follows:

ATGGTAGTTT TCGGTTTGA CTTTGGCACC ACATTCTCTA CGGTGTGTGT GTACAAGGAT 60  
 GGACGAGTTT TTTTATTCAA GCAGAATAAT TCGGCGTACA TCCCCACTTA CCTCTATCTC 120  
 TTCTCCGATT CTAACCACAT GACTTTTGGT TACGAGGCCG AATCACTGAT GAGTAATCTG 180  
 AAAGTTAAAG GTTCGTTTTA TAGAGATTTA AACCGTTGGG TGGGTTGCGA TTCGAGTAAC 240  
 CTCGACGCGT ACCTTGACCG TTTAAACCT CATTACTCGG TCCGCTTGGT TAAGATCGGC 300  
 TCTGGCTTGA ACGAACTGT TTCAATTGGA AACTTCGGGG GCACTGTAA GTCTGAGGCT 360  
 CATCTGCCAG GGTTGATAGC TCTCTTTATT AAGGCTGTCA TTAGTTGCGC GGAGGGCGCG 420  
 TTTGCGTGCA CTTGCACCGG GGTTATTTGT TCAGTACCTG CCAATTATGA TAGCGTTCAA 480  
 AGGAATTTCA CTGATCAGTG TGTTTCACTC AGCGGTTATC AGTGCGTATA TATGATCAAT 540  
 GAACCTTCAG CGGCTGCGCT ATCTGCGTGT AATTCGATTG GAAAGAAGTC CGCAAATTTG 600  
 GCTGTTTACG ATTTGCGTGG TGGGACCTTC GACGTGTCTA TCATTTTATA CCGCAACAAT 660  
 ACTTTTGTTG TGCGAGCTTC TGGAGGCGAT CTAAATCTCG GTGGAAGGGA TGTTGATCGT 720  
 GCGTTTCTCA CGCACCTCTT CTCTTTAACA TCGCTGGAAC CTGACCTCAC TTTGGATATC 780

TCGAATCTGA AAGAATCTTT ATCAAAAACG GACGCAGAGA TAGTTTACAC TTTGAGAGGT 840  
 GTCGATGGAA GAAAAGAAGA CGTTAGAGTA AACAAAAACA TTCTTACGTC GGTGATGCTC 900  
 CCCTACGTGA ACAGAACGCT TAAGATATTA GAGTCAACCT TAAAATCGTA TGCTAAGAGT 960  
 ATGAATGAGA GTGCGCGAGT TAAGTGCAT TTAGTGCTGA TAGGAGGATC TTCATATCTT 1020  
 CCTGGCCTGG CAGACGTACT AACGAAGCAT CAGAGCGTTG ATCGTATCTT AAGAGTTTCG 1080  
 GATCCTCGGG CTGCCGTGGC CGTCGGTTGC GCATTATATT CTTTCATGCCT CTCAGGATCT 1140  
 GGGGGGTTGC TACTGATCGA CTGTGCAGCT CACACTGTCG CTATAGCGGA CAGAAGTTGT 1200  
 CATCAAATCA TTTGCGCTCC AGCGGGGGCA CCGATCCCT TTTTCAGGAAG CATGCCTTTG 1260  
 TACTTAGCCA GGGTCAACAA GAACTCGCAG CGTGAAGTCG CCGTGTTTGA AGGGGAGTAC 1320  
 GTTAAGTGCC CTAAGAACAG AAAGATCTGT GGAGCAAATA TAAGATTTTT TGATATAGGA 1380  
 GTGACGGGTG ATTCGTACGC ACCCGTTACC TTCTATATGG ATTTCTCCAT TTCAAGCGTA 1440  
 GGAGCCGTTT CATTCGTGGT GAGAGGTCCT GAGGGTAAGC AAGTGTCCT CACTGGAAC 1500  
 CCAGCGTATA ACTTTTCGTC TGTGGCTCTC GGATCACGCA GTGTCCGAGA ATTGCATATT 1560  
 AGTTTAAATA ATAAAGTTTT TCTCGGTTTG CTTCTACATA GAAAGGCGGA TCGACGAATA 1620  
 CTTTTCATA AGGATGAAGC GATTTCGATAC GCCGATTCAA TTGATATCGC GGATGTGCTA 1680  
 AAGGAATATA AAAGTTACGC GGCCAGTGCC TTACCACCAG ACGAGGATGT CGAATTACTC 1740  
 CTGGGAAAGT CTGTTCAAAA AGTTTTACGG GGAAGCAGAC TGGAAGAAAT ACCTCTCTAG 1800

The heat shock 70 protein is believed to function as a chaperone protein and has an amino acid sequence corresponding to SEQ. ID. No. 9 as follows:

Met Val Val Phe Gly Leu Asp Phe Gly Thr Thr Phe Ser Thr Val Cys  
 1 5 10 15  
 Val Tyr Lys Asp Gly Arg Val Phe Ser Phe Lys Gln Asn Asn Ser Ala  
 20 25 30  
 Tyr Ile Pro Thr Tyr Leu Tyr Leu Phe Ser Asp Ser Asn His Met Thr  
 35 40 45  
 Phe Gly Tyr Glu Ala Glu Ser Leu Met Ser Asn Leu Lys Val Lys Gly  
 50 55 60  
 Ser Phe Tyr Arg Asp Leu Lys Arg Trp Val Gly Cys Asp Ser Ser Asn  
 65 70 75 80  
 Leu Asp Ala Tyr Leu Asp Arg Leu Lys Pro His Tyr Ser Val Arg Leu  
 85 90 95  
 Val Lys Ile Gly Ser Gly Leu Asn Glu Thr Val Ser Ile Gly Asn Phe  
 100 105 110

Gly Gly Thr Val Lys Ser Glu Ala His Leu Pro Gly Leu Ile Ala Leu  
115 120 125

Phe Ile Lys Ala Val Ile Ser Cys Ala Glu Gly Ala Phe Ala Cys Thr  
130 135 140

Cys Thr Gly Val Ile Cys Ser Val Pro Ala Asn Tyr Asp Ser Val Gln  
145 150 155 160

Arg Asn Phe Thr Asp Gln Cys Val Ser Leu Ser Gly Tyr Gln Cys Val  
165 170 175

Tyr Met Ile Asn Glu Pro Ser Ala Ala Ala Leu Ser Ala Cys Asn Ser  
180 185 190

Ile Gly Lys Lys Ser Ala Asn Leu Ala Val Tyr Asp Phe Gly Gly Gly  
195 200 205

Thr Phe Asp Val Ser Ile Ile Ser Tyr Arg Asn Asn Thr Phe Val Val  
210 215 220

Arg Ala Ser Gly Gly Asp Leu Asn Leu Gly Gly Arg Asp Val Asp Arg  
225 230 235 240

Ala Phe Leu Thr His Leu Phe Ser Leu Thr Ser Leu Glu Pro Asp Leu  
245 250 255

Thr Leu Asp Ile Ser Asn Leu Lys Glu Ser Leu Ser Lys Thr Asp Ala  
260 265 270

Glu Ile Val Tyr Thr Leu Arg Gly Val Asp Gly Arg Lys Glu Asp Val  
275 280 285

Arg Val Asn Lys Asn Ile Leu Thr Ser Val Met Leu Pro Tyr Val Asn  
290 295 300

Arg Thr Leu Lys Ile Leu Glu Ser Thr Leu Lys Ser Tyr Ala Lys Ser  
305 310 315 320

Met Asn Glu Ser Ala Arg Val Lys Cys Asp Leu Val Leu Ile Gly Gly  
325 330 335

Ser Ser Tyr Leu Pro Gly Leu Ala Asp Val Leu Thr Lys His Gln\_Ser  
340 345 350

Val Asp Arg Ile Leu Arg Val Ser Asp Pro Arg Ala Ala Val Ala Val  
355 360 365

Gly Cys Ala Leu Tyr Ser Ser Cys Leu Ser Gly Ser Gly Gly Leu Leu  
370 375 380

Leu Ile Asp Cys Ala Ala His Thr Val Ala Ile Ala Asp Arg Ser Cys  
385 390 395 400

His Gln Ile Ile Cys Ala Pro Ala Gly Ala Pro Ile Pro Phe Ser Gly  
405 410 415

Ser Met Pro Leu Tyr Leu Ala Arg Val Asn Lys Asn Ser Gln Arg Glu  
420 425 430

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Val Ala Val Phe Glu Gly Glu Tyr Val Lys Cys Pro Lys Asn Arg Lys  
435 440 445

Ile Cys Gly Ala Asn Ile Arg Phe Phe Asp Ile Gly Val Thr Gly Asp  
450 455 460

Ser Tyr Ala Pro Val Thr Phe Tyr Met Asp Phe Ser Ile Ser Ser Val  
465 470 475 480

Gly Ala Val Ser Phe Val Val Arg Gly Pro Glu Gly Lys Gln Val Ser  
485 490 495

Leu Thr Gly Thr Pro Ala Tyr Asn Phe Ser Ser Val Ala Leu Gly Ser  
500 505 510

Arg Ser Val Arg Glu Leu His Ile Ser Leu Asn Asn Lys Val Phe Leu  
515 520 525

Gly Leu Leu Leu His Arg Lys Ala Asp Arg Arg Ile Leu Phe Thr Lys  
530 535 540

Asp Glu Ala Ile Arg Tyr Ala Asp Ser Ile Asp Ile Ala Asp Val Leu  
545 550 555 560

Lys Glu Tyr Lys Ser Tyr Ala Ala Ser Ala Leu Pro Pro Asp Glu Asp  
565 570 575

Val Glu Leu Leu Leu Gly Lys Ser Val Gln Lys Val Leu Arg Gly Ser  
580 585 590

Arg Leu Glu Glu Ile Pro Leu  
595

and a molecular weight from about 63 to about 67 kDa, preferably about 65 kDa.

Another such DNA molecule (GLRaV-2 ORF4) includes nucleotides 11277-12932 of SEQ. ID. No. 1 and codes for a putative grapevine leafroll virus heat shock 90 protein. This DNA molecule comprises a nucleotide sequence corresponding to SEQ. ID. No. 10 as follows:

ATGTCGAATT ACTCCTGGGA AAGTCTGTTC AAAAAGTTTT ACGGGAAGC AGACTGGAAG 60

AAATACCTCT CTAGGAGCAT AGCAGCACAC TCAAGTGAAA TTAAACTCT ACCAGACATT 120

CGATTGTACG GCGGTAGGGT TGTAAGAAG TCCGAATTCG AATCAGCACT TCCTAATTCT 180

TTTGAACAGG AATTAGGACT GTTCATACTG AGCGAACGGG AAGTGGGATG GAGCAAATTA 240

TGCGGAATAA CGGTGGAAGA AGCAGCATAC GATCTTACGA ATCCCAAGGC TTATAAATTC 300

ACTGCCGAGA CATGTAGCCC GGATGTAAAA GGTGAAGGAC AAAAATACTC TATGGAAGAC 360

GTGATGAATT TCATGCGTTT ATCAAATCTG GATGTTAACG ACAAGATGCT GACGGAACAG 420

TGTTGGTCGC TGTCCAATTC ATGCGGTGAA TTGATCAACC CAGACGACAA AGGGCGATTC 480

GTGGCTCTCA CCTTTAAGGA CAGAGACACA GCTGATGACA CGGGTGCCGC CAACGTGGAA 540



TGTCGCGTGG GCGACTATCT AGTTTACGCT ATGTCCCTGT TTGAGCAGAG GACCCAAAAA 600  
 TCGCAGTCTG GCAACATCTC TCTGTACGAA AAGTACTGTG AATACATCAG GACCTACTTA 660  
 GGGAGTACAG ACCTGTTCTT CACAGCGCCG GACAGGATTC CGTTACTTAC GGGCATCCTA 720  
 TACGATTTTT GTAAGGAATA CAACGTTTTT TACTCGTCAT ATAAGAGAAA CGTCGATAAT 780  
 TTCAGATTCT TCTTGGCGAA TTATATGCCT TTGATATCTG ACGTCTTTGT CTTCCAGTGG 840  
 GTAAAACCCG CGCCGGATGT TCGGCTGCTT TTTGAGTTAA GTGCAGCGGA ACTAACGCTG 900  
 GAGGTTCCCA CACTGAGTTT GATAGATTCT CAAGTTGTGG TAGGTCATAT CTTAAGATAC 960  
 GTAGAATCCT ACACATCAGA TCCAGCCATC GACGCGTTAG AAGACAACT GGAAGCGATA 1020  
 CTGAAAAGTA GCAATCCCCG TCTATCGACA GCGCAACTAT GGGTTGGTTT CTTTTGTTAC 1080  
 TATGGTGAGT TTCGTACGGC TCAAAGTAGA GTAGTGCAA GACCAGGCGT ATACAAAACA 1140  
 CCTGACTCAG TGGGTGGATT TGAAATAAAC ATGAAAGATG TTGAGAAATT CTTCGATAAA 1200  
 CTTCAGAGAG AATTGCCTAA TGTATCTTTG CGGCGTCAGT TTAACGGAGC TAGAGCGCAT 1260  
 GAGGCTTTCA AAATATTTAA AAACGGAAAT ATAAGTTTCA GACCTATATC GCGTTTAAAC 1320  
 GTGCCTAGAG AGTTCTGGTA TCTGAACATA GACTACTTCA GGCACGCGAA TAGGTCCGGG 1380  
 TTAACCGAAG AAGAAATACT CATCCTAAAC AACATAAGCG TTGATGTTAG GAAGTTATGC 1440  
 GCTGAGAGAG CGTGCAATAC CCTACCTAGC GCGAAGCGCT TTAGTAAAAA TCATAAGAGT 1500  
 AATATACAAT CATCAGCCA AGAGCGGAGG ATTAAGACC CATTGGTAGT CCTGAAAGAC 1560  
 ACTTTATATG AGTTCCAACA CAAGCGTGCC GGTGGGGGT CTCGAAGCAC TCGAGACCTC 1620  
 GGGAGTCGTG CTGACCACGC GAAAGGAAGC GGTGA 1656

The heat shock 90 protein has an amino acid sequence corresponding to SEQ. ID. No. 11 as follows:

Met Ser Asn Tyr Ser Trp Glu Ser Leu Phe Lys Lys Phe Tyr Gly Glu  
 1 5 10 15  
 Ala Asp Trp Lys Lys Tyr Leu Ser Arg Ser Ile Ala Ala His Ser Ser  
 20 25 30  
 Glu Ile Lys Thr Leu Pro Asp Ile Arg Leu Tyr Gly Gly Arg Val Val  
 35 40 45  
 Lys Lys Ser Glu Phe Glu Ser Ala Leu Pro Asn Ser Phe Glu Gln Glu  
 50 55 60  
 Leu Gly Leu Phe Ile Leu Ser Glu Arg Glu Val Gly Trp Ser Lys Leu  
 65 70 75 80

Cys	Gly	Ile	Thr	Val	Glu	Glu	Ala	Ala	Tyr	Asp	Leu	Thr	Asn	Pro	Lys	
				85					90					95		
Ala	Tyr	Lys	Phe	Thr	Ala	Glu	Thr	Cys	Ser	Pro	Asp	Val	Lys	Gly	Glu	
			100					105					110			
Gly	Gln	Lys	Tyr	Ser	Met	Glu	Asp	Val	Met	Asn	Phe	Met	Arg	Leu	Ser	
		115					120					125				
Asn	Leu	Asp	Val	Asn	Asp	Lys	Met	Leu	Thr	Glu	Gln	Cys	Trp	Ser	Leu	
	130					135					140					
Ser	Asn	Ser	Cys	Gly	Glu	Leu	Ile	Asn	Pro	Asp	Asp	Lys	Gly	Arg	Phe	
145					150					155					160	
Val	Ala	Leu	Thr	Phe	Lys	Asp	Arg	Asp	Thr	Ala	Asp	Asp	Thr	Gly	Ala	
				165					170					175		
Ala	Asn	Val	Glu	Cys	Arg	Val	Gly	Asp	Tyr	Leu	Val	Tyr	Ala	Met	Ser	
			180					185					190			
Leu	Phe	Glu	Gln	Arg	Thr	Gln	Lys	Ser	Gln	Ser	Gly	Asn	Ile	Ser	Leu	
		195					200					205				
Tyr	Glu	Lys	Tyr	Cys	Glu	Tyr	Ile	Arg	Thr	Tyr	Leu	Gly	Ser	Thr	Asp	
	210					215					220					
Leu	Phe	Phe	Thr	Ala	Pro	Asp	Arg	Ile	Pro	Leu	Leu	Thr	Gly	Ile	Leu	
225					230					235					240	
Tyr	Asp	Phe	Cys	Lys	Glu	Tyr	Asn	Val	Phe	Tyr	Ser	Ser	Tyr	Lys	Arg	
				245					250					255		
Asn	Val	Asp	Asn	Phe	Arg	Phe	Phe	Leu	Ala	Asn	Tyr	Met	Pro	Leu	Ile	
			260					265					270			
Ser	Asp	Val	Phe	Val	Phe	Gln	Trp	Val	Lys	Pro	Ala	Pro	Asp	Val	Arg	
		275					280					285				
Leu	Leu	Phe	Glu	Leu	Ser	Ala	Ala	Glu	Leu	Thr	Leu	Glu	Val	Pro	Thr	
	290					295					300					
Leu	Ser	Leu	Ile	Asp	Ser	Gln	Val	Val	Val	Gly	His	Ile	Leu	Arg	Tyr	
305					310					315					320	
Val	Glu	Ser	Tyr	Thr	Ser	Asp	Pro	Ala	Ile	Asp	Ala	Leu	Glu	Asp	Lys	
				325					330					335		
Leu	Glu	Ala	Ile	Leu	Lys	Ser	Ser	Asn	Pro	Arg	Leu	Ser	Thr	Ala	Gln	
			340					345					350			
Leu	Trp	Val	Gly	Phe	Phe	Cys	Tyr	Tyr	Gly	Glu	Phe	Arg	Thr	Ala	Gln	
	355						360					365				
Ser	Arg	Val	Val	Gln	Arg	Pro	Gly	Val	Tyr	Lys	Thr	Pro	Asp	Ser	Val	
	370					375					380					
Gly	Gly	Phe	Glu	Ile	Asn	Met	Lys	Asp	Val	Glu	Lys	Phe	Phe	Asp	Lys	
385					390					395					400	

Leu Gln Arg Glu Leu Pro Asn Val Ser Leu Arg Arg Gln Phe Asn Gly  
405 410 415

Ala Arg Ala His Glu Ala Phe Lys Ile Phe Lys Asn Gly Asn Ile Ser  
420 425 430

Phe Arg Pro Ile Ser Arg Leu Asn Val Pro Arg Glu Phe Trp Tyr Leu  
435 440 445

Asn Ile Asp Tyr Phe Arg His Ala Asn Arg Ser Gly Leu Thr Glu Glu  
450 455 460

Glu Ile Leu Ile Leu Asn Asn Ile Ser Val Asp Val Arg Lys Leu Cys  
465 470 475 480

Ala Glu Arg Ala Cys Asn Thr Leu Pro Ser Ala Lys Arg Phe Ser Lys  
485 490 495

Asn His Lys Ser Asn Ile Gln Ser Ser Arg Gln Glu Arg Arg Ile Lys  
500 505 510

Asp Pro Leu Val Val Leu Lys Asp Thr Leu Tyr Glu Phe Gln His Lys  
515 520 525

Arg Ala Gly Trp Gly Ser Arg Ser Thr Arg Asp Leu Gly Ser Arg Ala  
530 535 540

Asp His Ala Lys Gly Ser Gly  
545 550

and a molecular weight from about 61 to about 65 kDa, preferably about 63 kDa.

Yet another DNA molecule of the present invention (GLRaV-2 ORF5) includes nucleotides 12844-13515 of SEQ. ID. No. 1 and codes for a diverged coat protein. This DNA molecule comprises a nucleotide sequence corresponding to SEQ. ID.

5 No. 12 as follows:

ATGAGTTCCA ACACAAGCGT GCCGGTTGGG GGTCTCGAAG CACTCGAGAC CTCGGGAGTC 60

GTGCTGACCA CGCGAAAGGA AGCGGTTGAT AAGTTTTTTA ATGAACTAAA AAACGAAAAT 120

TACTCATCAG TTGACAGCAG CCGATTAAGC GATTCGGAAG TAAAAGAAGT GTTAGAGAAA 180

AGTAAAGAAA GTTTCAAAAG CGAACTGGCC TCCACTGACG AGCACTTCGT CTACCACATT 240

ATATTTTTTCT TAATCCGATG TGCTAAGATA TCGACAAGTG AAAAGGTGAA GTACGTTGGT 300

AGTCATACGT ACGTGGTCGA CGGAAAAACG TACACCGTTC TTGACGCTTG GGTATTCAAC 360

ATGATGAAAA GTCTCACGAA GAAGTACAAA CGAGTGAATG GTCTGCGTGC GTTCTGTTGC 420

GCGTGCGAAG ATCTATATCT AACCGTCGCA CCAATAATGT CAGAACGCTT TAAGACTAAA 480

GCCGTAGGGA TGAAAGGTTT GCCTGTTGGA AAGGAATACT TAGGCGCCGA CTTTCTTTTCG 540

GGAAGTAGCA AACTGATGAG CGATCACGAC AGGGCGGTCT CCATCGTTGC AGCGAAAAAC 600

GCTGTCGATC GTAGCGCTTT CACGGGTGGG GAGAGAAAGA TAGTTAGTTT GTATGATCTA 660  
GGGAGGTACT AA 672

The diverged coat protein has an amino acid sequence corresponding to SEQ. ID. No. 13 as follows:

Met	Ser	Ser	Asn	Thr	Ser	Val	Pro	Val	Gly	Gly	Leu	Glu	Ala	Leu	Glu	1	5	10	15
Thr	Ser	Gly	Val	Val	Leu	Thr	Thr	Arg	Lys	Glu	Ala	Val	Asp	Lys	Phe	20	25	30	
Phe	Asn	Glu	Leu	Lys	Asn	Glu	Asn	Tyr	Ser	Ser	Val	Asp	Ser	Ser	Arg	35	40	45	
Leu	Ser	Asp	Ser	Glu	Val	Lys	Glu	Val	Leu	Glu	Lys	Ser	Lys	Glu	Ser	50	55	60	
Phe	Lys	Ser	Glu	Leu	Ala	Ser	Thr	Asp	Glu	His	Phe	Val	Tyr	His	Ile	65	70	75	80
Ile	Phe	Phe	Leu	Ile	Arg	Cys	Ala	Lys	Ile	Ser	Thr	Ser	Glu	Lys	Val	85	90	95	
Lys	Tyr	Val	Gly	Ser	His	Thr	Tyr	Val	Val	Asp	Gly	Lys	Thr	Tyr	Thr	100	105	110	
Val	Leu	Asp	Ala	Trp	Val	Phe	Asn	Met	Met	Lys	Ser	Leu	Thr	Lys	Lys	115	120	125	
Tyr	Lys	Arg	Val	Asn	Gly	Leu	Arg	Ala	Phe	Cys	Cys	Ala	Cys	Glu	Asp	130	135	140	
Leu	Tyr	Leu	Thr	Val	Ala	Pro	Ile	Met	Ser	Glu	Arg	Phe	Lys	Thr	Lys	145	150	155	160
Ala	Val	Gly	Met	Lys	Gly	Leu	Pro	Val	Gly	Lys	Glu	Tyr	Leu	Gly	Ala	165	170	175	
Asp	Phe	Leu	Ser	Gly	Thr	Ser	Lys	Leu	Met	Ser	Asp	His	Asp	Arg	Ala	180	185	190	
Val	Ser	Ile	Val	Ala	Ala	Lys	Asn	Ala	Val	Asp	Arg	Ser	Ala	Phe	Thr	195	200	205	
Gly	Gly	Glu	Arg	Lys	Ile	Val	Ser	Leu	Tyr	Asp	Leu	Gly	Arg	Tyr		210	215	220	

and a molecular weight from about 23 to about 27 kDa, preferably about 25 kDa.

- Another such DNA molecule (GLRaV-2 ORF6) includes nucleotides  
5 13584-14180 of SEQ. ID. No. 1 and codes for a grapevine leafroll virus coat protein.  
This DNA molecule comprises a nucleotide sequence corresponding to SEQ. ID. No. 14 as follows:

ATGGAGTTGA TGTCCGACAG CAACCTTAGC AACCTGGTGA TAACCGACGC CTCTAGTCTA 60  
AATGGTGTCTG ACAAGAAGCT TTTATCTGCT GAAGTTGAAA AAATGTTGGT GCAGAAAGGG 120  
GCTCCTAACG AGGGTATAGA AGTGGTGTTC GGTCTACTCC TTTACGCACT CGCGGCAAGA 180  
ACCACGTCTC CTAAGGTTCA GCGCGCAGAT TCAGACGTTA TATTTTCAAA TAGTTTCGGA 240  
GAGAGGAATG TGGTAGTAAC AGAGGGTGAC CTTAAGAAGG TACTCGACGG GTGTGCGCCT 300  
CTCACTAGGT TCACTAATAA ACTTAGAACG TTCGGTCGTA CTTTCACTGA GGCTTACGTT 360  
GACTTTTGTA TCGCGTATAA GCACAAATTA CCCCAACTCA ACGCCGCGGC GGAATTGGGG 420  
ATTCCAGCTG AAGATTCGTA CTTAGCTGCA GATTTTCTGG GTACTTGCCC GAAGCTCTCT 480  
GAATTACAGC AAAGTAGGAA GATGTTTCGG AGTATGTACG CTCTAAAAAC TGAAGGTGGA 540  
GTGGTAAATA CACCAGTGAG CAATCTGCGT CAGCTAGGTA GAAGGGAAGT TATGTAA 597

The coat protein has an amino acid sequence corresponding to SEQ. ID. No. 15 as follows:

Met Glu Leu Met Ser Asp Ser Asn Leu Ser Asn Leu Val Ile Thr Asp  
1 5 10 15  
Ala Ser Ser Leu Asn Gly Val Asp Lys Lys Leu Leu Ser Ala Glu Val  
20 25 30  
Glu Lys Met Leu Val Gln Lys Gly Ala Pro Asn Glu Gly Ile Glu Val  
35 40 45  
Val Phe Gly Leu Leu Leu Tyr Ala Leu Ala Ala Arg Thr Thr Ser Pro  
50 55 60  
Lys Val Gln Arg Ala Asp Ser Asp Val Ile Phe Ser Asn Ser Phe Gly  
65 70 75 80  
Glu Arg Asn Val Val Val Thr Glu Gly Asp Leu Lys Lys Val Leu Asp  
85 90 95  
Gly Cys Ala Pro Leu Thr Arg Phe Thr Asn Lys Leu Arg Thr Phe Gly  
100 105 110  
Arg Thr Phe Thr Glu Ala Tyr Val Asp Phe Cys Ile Ala Tyr Lys His  
115 120 125  
Lys Leu Pro Gln Leu Asn Ala Ala Ala Glu Leu Gly Ile Pro Ala Glu  
130 135 140  
Asp Ser Tyr Leu Ala Ala Asp Phe Leu Gly Thr Cys Pro Lys Leu Ser  
145 150 155 160  
Glu Leu Gln Gln Ser Arg Lys Met Phe Ala Ser Met Tyr Ala Leu Lys  
165 170 175

Thr Glu Gly Gly Val Val Asn Thr Pro Val Ser Asn Leu Arg Gln Leu  
180 185 190

Gly Arg Arg Glu Val Met  
195

and a molecular weight from about 20 to about 24 kDa, preferably about 22 kDa.

Another such DNA molecule (GLRaV-2 ORF7) includes nucleotides 14180-14665 of SEQ. ID. No. 1 and codes for a second undefined grapevine leafroll virus protein or polypeptide. This DNA molecule comprises a nucleotide sequence

5 corresponding to SEQ. ID. No. 16 as follows:

ATGGAAGATT ACGAAGAAAA ATCCGAATCG CTCATACTGC TACGCACGAA TCTGAACACT 60  
ATGCTTTTAG TGGTCAAGTC CGATGCTAGT GTAGAGCTGC CTAAACTACT AATTTGCGGT 120  
TACTTACGAG TGTCAGGACG TGGGGAGGTG ACGTGTTGCA ACCGTGAGGA ATTAACAAGA 180  
GATTTTGAGG GCAATCATCA TACGGTGATC CGTTCTAGAA TCATACAATA TGACAGCGAG 240  
TCTGCTTTTG AGGAATTCAA CAACTCTGAT TGCCTAGTGA AGTTTTTCCT AGAGACTGGT 300  
AGTGTCTTTT GGTTTTTTCCT TCGAAGTGAA ACCAAAGGTA GAGCGGTGCG ACATTTGCGC 360  
ACCTTCTTCG AAGCTAACAA TTTCTTCTTT GGATCGCATT GCGGTACCAT GGAGTATTGT 420  
TTGAAGCAGG TACTAACTGA AACTGAATCT ATAATCGATT CTTTTTGCGA AGAAAGAAAT 480  
CGTTAA 486

The second undefined grapevine leafroll virus protein or polypeptide has a deduced amino acid sequence corresponding to SEQ. ID. No. 17 as follows:

Met Glu Asp Tyr Glu Glu Lys Ser Glu Ser Leu Ile Leu Leu Arg Thr  
1 5 10 15

Asn Leu Asn Thr Met Leu Leu Val Val Lys Ser Asp Ala Ser Val Glu  
20 25 30

Leu Pro Lys Leu Leu Ile Cys Gly Tyr Leu Arg Val Ser Gly Arg Gly  
35 40 45

Glu Val Thr Cys Cys Asn Arg Glu Glu Leu Thr Arg Asp Phe Glu Gly  
50 55 60

Asn His His Thr Val Ile Arg Ser Arg Ile Ile Gln Tyr Asp Ser Glu  
65 70 75 80

Ser Ala Phe Glu Glu Phe Asn Asn Ser Asp Cys Val Val Lys Phe Phe  
85 90 95

Leu Glu Thr Gly Ser Val Phe Trp Phe Phe Leu Arg Ser Glu Thr Lys  
100 105 110

Gly Arg Ala Val Arg His Leu Arg Thr Phe Phe Glu Ala Asn Asn Phe  
 115 120 125  
 Phe Phe Gly Ser His Cys Gly Thr Met Glu Tyr Cys Leu Lys Gln Val  
 130 135 140  
 Leu Thr Glu Thr Glu Ser Ile Ile Asp Ser Phe Cys Glu Glu Arg Asn  
 145 150 155 160  
 Arg

and a molecular weight from about 17 to about 21 kDa, preferably about 19 kDa.

Yet another such DNA molecule (GLRaV-2 ORF8) includes nucleotides 14667-15284 of SEQ. ID. No. 1 and codes for a third undefined grapevine leafroll virus protein or polypeptide. This DNA molecule comprises a nucleotide sequence  
 5 corresponding to SEQ. ID. No. 18 as follows:

ATGAGGGTTA TAGTGTCTCC TTATGAAGCT GAAGACATTC TGAAAAGATC GACTGACATG 60  
 TTACGAAACA TAGACAGTGG GGTCTTGAGC ACTAAAGAAT GTATCAAGGC ATTCTCGACG 120  
 ATAACGCGAG ACCTACATTG TGCGAAGGCT TCCTACCAGT GGGGTGTTGA CACTGGGTTA 180  
 TATCAGCGTA ATTGCGCTGA AAAACGTTTA ATTGACACGG TGGAGTCAAA CATACGGTTG 240  
 GCTCAACCTC TCGTGCGTGA AAAAGTGGCG GTTCATTTTT GTAAGGATGA ACCAAAAGAG 300  
 CTAGTAGCAT TCATCACGCG AAAGTACGTG GAACTCACGG GCGTGGGAGT GAGAGAAGCG 360  
 GTGAAGAGGG AAATGCGCTC TCTTACCAA ACAGTTTTAA ATAAATGTC TTTGGAAATG 420  
 GCGTTTTACA TGTCACCACG AGCGTGGAAC AACGCTGAAT GGTTAGAACT AAAATTTTCA 480  
 CCTGTGAAAA TCTTTAGAGA TCTGCTATTA GACGTGGAAC CGCTCAACGA ATTGTGCGCC 540  
 GAAGATGATG TTCACGTCGA CAAAGTAAAT GAGAATGGGG ACGAAAATCA CGACCTCGAA 600  
 CTCCAAGACG AATGTTAA 618

The third undefined protein or polypeptide has a deduced amino acid sequence corresponding to SEQ. ID. No. 19 as follows:

Met Arg Val Ile Val Ser Pro Tyr Glu Ala Glu Asp Ile Leu Lys Arg  
 1 5 10 15  
 Ser Thr Asp Met Leu Arg Asn Ile Asp Ser Gly Val Leu Ser Thr Lys  
 20 25 30  
 Glu Cys Ile Lys Ala Phe Ser Thr Ile Thr Arg Asp Leu His Cys Ala  
 35 40 45

Lys	Ala	Ser	Tyr	Gln	Trp	Gly	Val	Asp	Thr	Gly	Leu	Tyr	Gln	Arg	Asn	50	55	60
Cys	Ala	Glu	Lys	Arg	Leu	Ile	Asp	Thr	Val	Glu	Ser	Asn	Ile	Arg	Leu	65	70	75
Ala	Gln	Pro	Leu	Val	Arg	Glu	Lys	Val	Ala	Val	His	Phe	Cys	Lys	Asp	85	90	95
Glu	Pro	Lys	Glu	Leu	Val	Ala	Phe	Ile	Thr	Arg	Lys	Tyr	Val	Glu	Leu	100	105	110
Thr	Gly	Val	Gly	Val	Arg	Glu	Ala	Val	Lys	Arg	Glu	Met	Arg	Ser	Leu	115	120	125
Thr	Lys	Thr	Val	Leu	Asn	Lys	Met	Ser	Leu	Glu	Met	Ala	Phe	Tyr	Met	130	135	140
Ser	Pro	Arg	Ala	Trp	Lys	Asn	Ala	Glu	Trp	Leu	Glu	Leu	Lys	Phe	Ser	145	150	155
Pro	Val	Lys	Ile	Phe	Arg	Asp	Leu	Leu	Leu	Asp	Val	Glu	Thr	Leu	Asn	165	170	175
Glu	Leu	Cys	Ala	Glu	Asp	Asp	Val	His	Val	Asp	Lys	Val	Asn	Glu	Asn	180	185	190
Gly	Asp	Glu	Asn	His	Asp	Leu	Glu	Leu	Gln	Asp	Glu	Cys				195	200	205

and a molecular weight from about 22 to about 26 kDa, preferably about 24 kDa.

Another DNA molecule of the present invention (GLRaV-2 3' UTR) includes nucleotides 15285-15500 of SEQ. ID. No. 1 and comprises a nucleotide sequence corresponding to SEQ. ID. No. 23 as follows:

ACATTGGTTA	AGTTTAACGA	AAATGATTAG	TAAATAATAA	ATCGAACGTG	GGTGTATCTA	60
CCTGACGTAT	CAACTTAAGC	TGTTACTGAG	TAATTAAACC	AACAAGTGTT	GGTGTAATGT	120
GTATGTTGAT	GTAGAGAAAA	ATCCGTTTGT	AGAACGGTGT	TTTTCTCTTC	TTTATTTTTA	180
AAAAAAAAAT	AAAAAAAAAA	AAAAAAAAAGC	GGCCGC			216

5 Also encompassed by the present invention are fragments of the DNA molecules of the present invention. Suitable fragments capable of imparting grapevine leafroll resistance to grape plants are constructed by using appropriate restriction sites, revealed by inspection of the DNA molecule's sequence, to: (i) insert an interposon (Felley et al., "Interposon Mutagenesis of Soil and Water Bacteria: a Family of DNA

10 Fragments Designed for in vitro Insertion Mutagenesis of Gram-negative Bacteria," Gene, 52:147-15 (1987), which is hereby incorporated by reference) such that truncated



forms of the grapevine leafroll virus coat polypeptide or protein, that lack various amounts of the C-terminus, can be produced or (ii) delete various internal portions of the protein. Alternatively, the sequence can be used to amplify any portion of the coding region, such that it can be cloned into a vector supplying both transcription and translation start signals.

Suitable DNA molecules are those that hybridize to a DNA molecule comprising a nucleotide sequence of at least 15 continuous bases of SEQ. ID. No. 1 under stringent conditions characterized by a hybridization buffer comprising 0.9M sodium citrate ("SSC") buffer at a temperature of 37°C and remaining bound when subject to washing with SSC buffer at 37°C; and preferably in a hybridization buffer comprising 20% formamide in 0.9M saline/0.9M SSC buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2x SSC buffer at 42°C.

Variants may also (or alternatively) be modified by, for example, the deletion or addition of nucleotides that have minimal influence on the properties, secondary structure and hydrophobic nature of the encoded polypeptide. For example, the nucleotides encoding a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The nucleotide sequence may also be altered so that the encoded polypeptide is conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

The protein or polypeptide of the present invention is preferably produced in purified form (preferably, at least about 80%, more preferably 90%, pure) by conventional techniques. Typically, the protein or polypeptide of the present invention is isolated by ~~lysing and sonication~~. After washing, the lysate pellet is resuspended in buffer containing Tris-HCl. During dialysis, a precipitate forms from this protein solution. The solution is centrifuged, and the pellet is washed and resuspended in the buffer containing Tris-HCl. Proteins are resolved by electrophoresis through an SDS 12% polyacrylamide gel.

The DNA molecule encoding the grapevine leafroll virus (type 2) protein or polypeptide of the present invention can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the

necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccinia virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC184, pUC8, pUC9, pUC18, pUC19, pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see Studier et. al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology, vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1982), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria or transformed via particle bombardment (i.e. biolistics). The expression elements of these vectors vary in their

strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA ("mRNA") translation).

5 Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic  
10 signals may not be recognized in or may not function in a procaryotic system, and, further, procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eucaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence  
15 of mRNA that is located before the start codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473  
20 (1979), which is hereby incorporated by reference.

Promoters vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, ~~any one of a number of suitable~~  
25 promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the  $P_R$  and  $P_L$  promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other  
30 synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operons, the addition of specific inducers is necessary for efficient transcription of the inserted DNA.

For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno ("SD") sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecules encoding the various grapevine leafroll virus (type 2) proteins or polypeptides, as described above, have been cloned into an expression system, they are ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention also relates to RNA molecules which encode the various grapevine leafroll virus (type 2) proteins or polypeptides described above. The transcripts can be synthesized using the host cells of the present invention by any of the conventional techniques. The mRNA can be translated either *in vitro* or *in vivo*. Cell-free systems typically include wheat-germ or reticulocyte extracts. *In vivo* translation can be effected, for example, by microinjection into frog oocytes.

One aspect of the present invention involves using one or more of the above DNA molecules encoding the various proteins or polypeptides of a grapevine leafroll virus (type 2) to transform grape plants in order to impart grapevine leafroll resistance to the plants. The mechanism by which resistance is imparted is not known. In one hypothetical mechanism, the transformed plant can express a protein or polypeptide of grapevine leafroll virus (type 2), and, when the transformed plant is inoculated by a

grapevine leafroll virus, such as GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, or GLRaV-6, or combinations of these, the expressed protein or polypeptide prevents translation of the viral DNA.

In this aspect of the present invention the subject DNA molecule  
5 incorporated in the plant can be constitutively expressed. Alternatively, expression can be regulated by a promoter which is activated by the presence of grapevine leafroll virus. Suitable promoters for these purposes include those from genes expressed in response to grapevine leafroll virus infiltration.

The isolated DNA molecules of the present invention can be utilized to  
10 impart grapevine leafroll virus resistance for a wide variety of grapevine plants. The DNA molecules are particularly well suited to imparting resistance to *Vitis* scion or rootstock cultivars. Scion cultivars which can be protected include those commonly referred to as Table or Raisin Grapes, such as Alden, Almeria, Anab-E-Shahi, Autumn Black, Beauty Seedless, Black Corinth, Black Damascus, Black Malvoisie, Black Prince,  
15 Blackrose, Bronx Seedless, Burgrave, Calmeria, Campbell Early, Canner, Cardinal, Catawba, Christmas, Concord, Dattier, Delight, Diamond, Dizmar, Duchess, Early Muscat, Emerald Seedless, Emperor, Exotic, Ferdinand de Lesseps, Fiesta, Flame seedless, Flame Tokay, Gasconade, Gold, Himrod, Hunisa, Hussiene, Isabella, Italia, July Muscat, Khandahar, Katta, Kourgane, Kishmishi, Loose Perlette, Malaga, Monukka,  
20 Muscat of Alexandria, Muscat Flame, Muscat Hamburg, New York Muscat, Niabell, Niagara, Olivette blanche, Ontario, Pierce, Queen, Red Malaga, Ribier, Rish Baba, Romulus, Ruby Seedless, Schuyler, Seneca, Suavis (IP 365), Thompson seedless, and Thomuscat. They also include those used in wine production, such as Aleatico, Alicante Bouschet, Aligote, Alvarelhao, Aramon, Baco blanc (22A), Burger, Cabernet franc,  
25 Cabernet, Sauvignon, Calzin, Carignane, Charbono, Chardonnay, Chasselas dore, Chenin blanc, Clairette blanche, Early Burgundy, Emerald Riesling, Feher Szagos, Fernao Pires, Flora, French Colombard, Fresia, Furmint, Gamay, Gewurztraminer, Grand noir, Gray Riesling, Green Hungarian, Green Veltliner, Grenache, Grillo, Helena, Inzolia, Lagrein, Lambrusco de Salamino, Malbec, Malvasia bianca, Mataro, Melon, Merlot, Meunier,  
30 Mission, Montua de Pilas, Muscadelle du Bordelais, Muscat blanc, Muscat Ottonel, Muscat Saint-Vallier, Nebbiolo, Nebbiolo fino, Nebbiolo Lampia, Orange Muscat, Palomino, Pedro Ximenes, Petit Bouschet, Petite Sirah, Peverella, Pinot noir, Pinot Saint-George, Primitivo di Gioia, Red Veltliner, Refosco, Rkatsiteli, Royalty, Rubired, Ruby Cabernet, Saint-Emilion, Saint Macaire, Salvador, Sangiovese, Sauvignon blanc,

Sauvignon gris, Sauvignon vert, Scarlet, Seibel 5279, Seibel 9110, Seibel 13053, Semillon, Servant, Shiraz, Souzao, Sultana Crimson, Sylvaner, Tannat, Teroldico, Tinta Madeira, Tinto cao, Touriga, Traminer, Trebbiano Toscano, Trousseau, Valdepenas, Viognier, Walschriesling, White Riesling, and Zinfandel. Rootstock cultivars which can  
5 be protected include Couderc 1202, Couderc 1613, Couderc 1616, Couderc 3309, Dog Ridge, Foex 33 EM, Freedom, Ganzin 1 (A x R #1), Harmony, Kober 5BB, LN33, Millardet & de Grasset 41B, Millardet & de Grasset 420A, Millardet & de Grasset 101-14, Oppenheim 4 (SO4), Paulsen 775, Paulsen 1045, Paulsen 1103, Richter 99, Richter 110, Riparia Gloire, Ruggeri 225, Saint-George, Salt Creek, Teleki 5A, Vitis rupestris  
10 Constantia, *Vitis californica*, and *Vitis girdiana*.

There exists an extensive similarity in the hsp70-related sequence regions of GLRaV-2 and other closteroviruses, such as tristeza virus and beet yellows virus. Consequently, the GLRaV-2 hsp70-related gene can also be used to produce transgenic plants or cultivars other than grape, such as citrus or sugar beet, which are resistant to  
15 closteroviruses other than grapevine leafroll, such as tristeza virus and beet yellows virus.

Suitable citrus cultivars include lemon, lime, orange, grapefruit, pineapple, tangerine, and the like, such as Joppa, Maltaise Ovale, Parson (Parson Brown), Pera, Pineapple, Queen, Shamouti, Valencia, Tenerife, Imperial Doblefina, Washington Sanguine, Moro, Sanguinello Moscato, Spanish Sanguinelli, Tarocco, Atwood,  
20 Australian, Bahia, Baiana, Cram, Dalmau, Eddy, Fisher, Frost Washington, Gillette, LengNavelina, Washington, Satsuma Mandarin, Dancy, Robinson, Ponkan, Duncan, Marsh, Pink Marsh, Ruby Red, Red Seedless, Smooth Seville, Orlando Tangelo, Eureka, Lisbon, Meyer Lemon, Rough Lemon, Sour Orange, Persian Lime, West Indian Lime, Bearss, Sweet Lime, Troyer Citrange, and Citrus Trifoliata. Each of these citrus cultivars  
25 is suitable for producing transgenic citrus plants resistant to tristeza virus.

The economically important species of sugar beet is *Beta vulgaris L.*, which has four important cultivar types: sugar beet, table beet, fodder beet, and Swiss chard. Each of these beet cultivars is suitable for producing transgenic beet plants resistant to beet yellows virus, as described above.

30 Because GLRaV-2 has been known to infect tobacco plants (e.g., *Nicotiana benthamiana*), it is also desirable to produce transgenic tobacco plants which are resistant to grapevine leafroll viruses, such as GLRaV-2.

00674186 074400  
007720 98412580

Plant tissue suitable for transformation include leaf tissue, root tissue, meristems, zygotic and somatic embryos, and anthers. It is particularly preferred to utilize embryos obtained from anther cultures.

The expression system of the present invention can be used to transform  
5 virtually any plant tissue under suitable conditions. Tissue cells transformed in accordance with the present invention can be grown *in vitro* in a suitable medium to impart grapevine leafroll virus resistance. Transformed cells can be regenerated into whole plants such that the protein or polypeptide imparts resistance to grapevine leafroll virus in the intact transgenic plants. In either case, the plant cells transformed with the recombinant DNA expression  
10 system of the present invention are grown and caused to express that DNA molecule to produce one of the above-described grapevine leafroll virus proteins or polypeptides and, thus, to impart grapevine leafroll virus resistance.

In producing transgenic plants, the DNA construct in a vector described above can be microinjected directly into plant cells by use of micropipettes to transfer mechanically  
15 the recombinant DNA. Crossway, Mol. Gen. Genetics, 202:179-85 (1985), which is hereby incorporated by reference. The genetic material may also be transferred into the plant cell using polyethylene glycol. Krens, et al., Nature, 296:72-74 (1982), which is hereby incorporated by reference.

One technique of transforming plants with the DNA molecules in  
20 accordance with the present invention is by contacting the tissue of such plants with an inoculum of a bacteria transformed with a vector comprising a gene in accordance with the present invention which imparts grapevine leafroll resistance. Generally, this procedure involves inoculating the plant tissue with a suspension of bacteria and  
25 incubating the tissue for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species of such bacterium include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* (e.g., strains C58, LBA4404, or EHA105) is particularly useful due to its well-known ability to transform plants.

30 Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *A. tumefaciens* or the Ri plasmid of *A. rhizogenes*. The Ti or Ri plasmid is transmitted to plant cells on infection by *Agrobacterium* and is stably integrated into the plant genome. J. Schell, Science, 237:1176-83 (1987), which is hereby incorporated by reference.

After transformation, the transformed plant cells must be regenerated.

Plant regeneration from cultured protoplasts is described in Evans et al., Handbook of Plant Cell Cultures, Vol. 1: (MacMillan Publishing Co., New York, 1983); and Vasil I.R. (ed.), Cell Culture and Somatic Cell Genetics of Plants, Acad. Press, Orlando, Vol. I, 1984, and Vol. III (1986), which are hereby incorporated by reference.

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to, all major species of sugarcane, sugar beets, cotton, fruit trees, and legumes.

Means for regeneration vary from species to species of plants, but generally ~~a~~ suspension of transformed protoplasts or a petri plate containing explants is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced in the callus tissue. These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is usually reproducible and repeatable.

After the expression cassette is stably incorporated in transgenic plants, it can be transferred to other plants by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

Once transgenic plants of this type are produced, the plants themselves can be cultivated in accordance with conventional procedure so that the DNA construct is present in the resulting plants. Alternatively, transgenic seeds are recovered from the transgenic plants. These seeds can then be planted in the soil and cultivated using conventional procedures to produce transgenic plants.

Another approach to transforming plant cells with a gene which imparts resistance to pathogens is particle bombardment (also known as biolistic transformation) of the host cell. This can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., and in Emerschad et al., "Somatic Embryogenesis and Plant Development from Immature Zygotic Embryos of Seedless Grapes (*Vitis vinifera*)," Plant Cell Reports, 14:6-12 (1995) ("Emerschad (1995)"), which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under



conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the heterologous DNA.

Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the vector and heterologous DNA) can also be propelled into plant cells.

Once a grape plant tissue, citrus plant tissue, beet plant tissue, or tobacco plant tissue is transformed in accordance with the present invention, the transformed tissue is regenerated to form a transgenic plant. Generally, regeneration is accomplished by culturing transformed tissue on medium containing the appropriate growth regulators and nutrients to allow for the initiation of shoot meristems. Appropriate antibiotics are added to the regeneration medium to inhibit the growth of *Agrobacterium* and to select for the development of transformed cells. Following shoot initiation, shoots are allowed to develop tissue culture and are screened for marker gene activity.

The DNA molecules of the present invention can be made capable of transcription to a messenger RNA, which, although encoding for a grapevine leafroll virus (type 2) protein or polypeptide, does not translate to the protein. This is known as RNA-mediated resistance. When a *Vitis* scion or rootstock cultivar, or a citrus, beet, or tobacco cultivar, is transformed with such a DNA molecule, the DNA molecule can be transcribed under conditions effective to maintain the messenger RNA in the plant cell at low level density readings. Density readings of between 15 and 50 using a Hewlet ScanJet and Image Analysis Program are preferred.

A portion of one or more DNA molecules of the present invention as well as other DNA molecules can be used in a transgenic grape plant, citrus plant, beet plant, or tobacco plant in accordance with U.S. Patent Application Serial No. 09/025,635, which is hereby incorporated herein by reference.

The grapevine leafroll virus (type 2) protein or polypeptide of the present invention can also be used to raise antibodies or binding portions thereof or probes. The antibodies can be monoclonal or polyclonal.

Monoclonal antibody production may be effected by techniques which are well-known in the art. Basically, the process involves first obtaining immune cells (lymphocytes) from the spleen of a mammal (e.g., mouse) which has been previously immunized with the antigen of interest either *in vivo* or *in vitro*. The antibody-secreting lymphocytes are then fused with (mouse) myeloma cells or transformed cells, which are

capable of replicating indefinitely in cell culture, thereby producing an immortal, immunoglobulin-secreting cell line. The resulting fused cells, or hybridomas, are cultured, and the resulting colonies screened for the production of the desired monoclonal antibodies. Colonies producing such antibodies are cloned, and grown either *in vivo* or *in vitro* to produce large quantities of antibody. A description of the theoretical basis and practical methodology of fusing such cells is set forth in Kohler and Milstein, Nature, 256:495 (1975), which is hereby incorporated by reference.

Mammalian lymphocytes are immunized by *in vivo* immunization of the animal (e.g., a mouse) with the protein or polypeptide of the present invention. Such immunizations are repeated as necessary at intervals of up to several weeks to obtain a sufficient titer of antibodies. Following the last antigen boost, the animals are sacrificed and spleen cells removed.

Fusion with mammalian myeloma cells or other fusion partners capable of replicating indefinitely in cell culture is effected by standard and well-known techniques, for example, by using polyethylene glycol ("PEG") or other fusing agents. (See Milstein and Kohler, Eur. J. Immunol., 6:511 (1976), which is hereby incorporated by reference.) This immortal cell line, which is preferably murine, but may also be derived from cells of other mammalian species, including but not limited to rats and humans, is selected to be deficient in enzymes necessary for the utilization of certain nutrients, to be capable of rapid growth, and to have good fusion capability. Many such cell lines are known to those skilled in the art, and others are regularly described.

Procedures for raising polyclonal antibodies are also well known. Typically, such antibodies can be raised by administering the protein or polypeptide of the present invention subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100  $\mu$ l per site at six different sites. Each injected material will contain synthetic surfactant adjuvant pluronic polyols, or pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. Ultimately, the rabbits are euthenized with pentobarbital 150 mg/Kg IV. This and other procedures for raising

polyclonal antibodies are disclosed in Harlow et. al., editors, Antibodies: A Laboratory Manual (1988), which is hereby incorporated by reference.

In addition to utilizing whole antibodies, binding portions of such antibodies can be used. Such binding portions include Fab fragments, F(ab')<sub>2</sub> fragments, and Fv fragments. These antibody fragments can be made by conventional procedures, such as proteolytic fragmentation procedures, as described in Goding, Monoclonal Antibodies: Principles and Practice, New York:Academic Press, pp. 98-118 (1983), which is hereby incorporated by reference.

The present invention also relates to probes found either in nature or prepared synthetically by recombinant DNA procedures or other biological procedures. Suitable probes are molecules which bind to grapevine leafroll (type 2) viral antigens identified by the monoclonal antibodies of the present invention. Such probes can be, for example, proteins, peptides, lectins, or nucleic acid probes.

The antibodies or binding portions thereof or probes can be administered to grapevine leafroll virus infected scion cultivars or rootstock cultivars. Alternatively, at least the binding portions of these antibodies can be sequenced, and the encoding DNA synthesized. The encoding DNA molecule can be used to transform plants together with a promoter which causes expression of the encoded antibody when the plant is infected by grapevine leafroll virus. In either case, the antibody or binding portion thereof or probe will bind to the virus and help prevent the usual leafroll response.

Antibodies raised against the GLRaV-2 proteins or polypeptides of the present invention or binding portions of these antibodies can be utilized in a method for detection of grapevine leafroll virus in a sample of tissue, such as tissue (e.g., scion or rootstock) from a grape plant or tobacco plant. Antibodies or binding portions thereof suitable for use in the detection method include those raised against a helicase, a methyltransferase, a papain-like protease, an RNA-dependent RNA polymerase, a heat shock 70 protein, a heat shock 90 protein, a coat protein, a diverged coat protein, or other proteins or polypeptides in accordance with the present invention. Any reaction of the sample with the antibody is detected using an assay system which indicates the presence of grapevine leafroll virus in the sample. A variety of assay systems can be employed, such as enzyme-linked immunosorbent assays, radioimmunoassays, gel diffusion precipitin reaction assays, immunodiffusion assays, agglutination assays, fluorescent immunoassays, protein A immunoassays, or immunoelectrophoresis assays.

Alternatively, grapevine leafroll virus can be detected in such a sample using a nucleotide sequence of the DNA molecule, or a fragment thereof, encoding for a protein or polypeptide of the present invention. The nucleotide sequence is provided as a probe in a nucleic acid hybridization assay or a gene amplification detection procedure (e.g., using a polymerase chain reaction procedure). The nucleic acid probes of the present invention may be used in any nucleic acid hybridization assay system known in the art, including, but not limited to, Southern blots (Southern, E.M., "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis," J. Mol. Biol., 98:503-17 (1975), which is hereby incorporated by reference), Northern blots (Thomas, P.S., "Hybridization of Denatured RNA and Small DNA Fragments Transferred to Nitrocellulose," Proc. Nat'l Acad. Sci. USA, 77:5201-05 (1980), which is hereby incorporated by reference), and Colony blots (Grunstein, M., et al., "Colony Hybridization: A Method for the Isolation of Cloned cDNAs that Contain a Specific Gene," Proc. Nat'l Acad. Sci. USA, 72:3961-65 (1975), which is hereby incorporated by reference). Alternatively, the probes can be used in a gene amplification detection procedure (e.g., a polymerase chain reaction). Erlich, H.A., et. al., "Recent Advances in the Polymerase Chain Reaction," Science 252:1643-51 (1991), which is hereby incorporated by reference. Any reaction with the probe is detected so that the presence of a grapevine leafroll virus in the sample is indicated. Such detection is facilitated by providing the probe of the present invention with a label. Suitable labels include a radioactive compound, a fluorescent compound, a chemiluminescent compound, an enzymatic compound, or other equivalent nucleic acid labels.

Depending upon the desired scope of detection, it is possible to utilize probes having nucleotide sequences that correspond with conserved or variable regions of the ORF or UTR. For example, to distinguish a grapevine leafroll virus from other related viruses (e.g., other closteroviruses), it is desirable to use probes which contain nucleotide sequences that correspond to sequences more highly conserved among all grapevine leafroll viruses. Also, to distinguish between different grapevine leafroll viruses (i.e., GLRaV-2 from GLRaV-1, GLRaV-3, GLRaV-4, GLRaV-5, and GLRaV-6), it is desirable to utilize probes containing nucleotide sequences that correspond to sequences less highly conserved among the different grapevine leafroll viruses.

Nucleic acid (DNA or RNA) probes of the present invention will hybridize to complementary GLRaV-2 nucleic acid under stringent conditions. Generally, stringent conditions are selected to be about 50°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under

defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. The  $T_m$  is dependent upon the solution conditions and the base composition of the probe, and may be calculated using the following equation:

$$\begin{aligned} T_m = & 79.8^{\circ}\text{C} + (18.5 \times \text{Log}[\text{Na}^+]) \\ & + (58.4^{\circ}\text{C} \times \%[\text{G}+\text{C}]) \\ & - (820 / \# \text{bp in duplex}) \\ & - (0.5 \times \% \text{ formamide}) \end{aligned}$$

Nonspecific binding may also be controlled using any one of a number of known techniques such as, for example, blocking the membrane with protein-containing solutions, addition of heterologous RNA, DNA, and SDS to the hybridization buffer, and treatment with RNase. Wash conditions are typically performed at or below stringency. Generally, suitable stringent conditions for nucleic acid hybridization assays or gene amplification detection procedures are as set forth above. More or less stringent conditions may also be selected.

## EXAMPLES

The following examples are provided to illustrate embodiments of the present invention but are by no means intended to limit its scope.

### Example 1 - Northern Hybridization

Specificity of the selected clones was confirmed by Northern hybridization. Northern hybridization was performed after electrophoresis of the dsRNA of GLRaV-2 in 1% agarose non-denaturing condition gel. The agarose gel was denatured by soaking in 50 mM NaOH containing 0.4 M NaCl for 30 min, and then neutralized with 0.1 M Tris-HCl (PH7.5 ) containing 0.5 M NaCl for another 30 min. RNA was sandwich blotted overnight onto Genescreen™ plus membrane (Dupont NEN Research Product) in 10 X SSC buffer and hybridized as described by the manufacturer's instructions (DuPont, NEN).

### Example 2 - Sequencing and Computer Assisted Nucleotide and Amino Acid Sequence Analysis

DNA inserts were sequenced in pBluescript SK+ by using T3 and T7 universal primers for the terminal region sequence and additional oligonucleotide primers

designed according to the known sequence for the internal region sequence. Purification of plasmid DNA was performed by a modified mini alkaline-lysis/PEG precipitation procedure described by the manufacturer (Applied Biosystems, Inc.). Nucleotide sequencing was performed on both strands of cDNA by using ABI TaqDyeDeoxy Terminator Cycle  
5 Sequencing Kit (Applied Biosystems, Inc.). Automatic sequencing was performed on an ABI373 Automated Sequencer (Applied Biosystems, Inc.) at Cornell University, Geneva, NY.

The nucleotide sequences of GLRaV-2 were assembled and analyzed with the programs of EditSeq and SeqMan, respectively, of DNASTAR package (Madison, WI).  
10 Amino acid sequences deduced from nucleotide sequences and its encoding open reading frames were conducted using the MapDraw program. Multiple alignments of amino acid sequences, identification of consensus amino acid sequences, and generation of phylogenetic trees were performed using the Clustal method in the MegAlign program. The nucleotide and amino acid sequences of other closteroviruses were obtained with the Entrez Program;  
15 and sequence comparisons with nonredundant databases were searched with the Blast Program from the National Center for Biotechnology Information.

### **Example 3 - Isolation of dsRNA**

Several vines of GLRaV-2 infected *Vitis vinifera* cv Pinot Noir that originated from a central New York vineyard served as the source for dsRNA isolation and cDNA cloning. dsRNA was extracted from phloem tissue of infected grapevines according to the method described by Hu et al., "Characterization of Closterovirus-Like Particles Associated with Grapevine Leafroll Disease," J. Phytopathology 128:1-14 (1990), which is hereby  
25 incorporated by reference. Purification of the high molecular weight dsRNA (ca 15 kb) was carried out by electrophoretic separation of the total dsRNA on a 0.7% low melting point agarose gel and extraction by phenol/chloroform following the method described by Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Sping Harbor Laboratory Press, New York (1989) , which is hereby incorporated by reference.  
30 Concentration of dsRNA was estimated with UV fluorescent density of an ethidium bromide stained dsRNA band in comparison with a known concentration of DNA marker.

#### **Example 4 - cDNA Synthesis and Cloning**

cDNA synthesis was performed following the method initially described by Jelkmann et al., "Cloning of Four Plant Viruses From Small Quantities of Double-Stranded RNA," Phytopathology 79:1250-53 (1989) and modified by Ling et al., "The Coat Protein Gene of Grapevine Leafroll Associated Closterovirus-3: Cloning, Nucleotide Sequencing and Expression in Transgenic Plants," Arch. Virology 142:1101-16 (1997), both of which are hereby incorporated by reference. About 100 ng of high molecular weight dsRNA purified from low melting agarose gel was denatured in 20 mM methylmercuric hydroxide and incubated at room temperature for 10 min with 350 ng of random primers. First strand cDNA was synthesized by using avian myeloblastosis virus (AMV) reverse transcriptase. Second strand cDNA was obtained by using RNase H and *E.coli* DNA polymerase I. Double-stranded cDNA was blunt ended with T4 DNA polymerase and ligated with EcoR I adapters. The cDNA, which had EcoR I adapters at the ends, was activated by kinase reaction and ligated into Lambda ZAP II/EcoR I prepared arms following the manufacturer's instruction (Stratagene). The recombinant DNA was then packaged *in vitro* to Gigapack® II packaging extract (Stratagene). The packaged phage particles were amplified and titered according to the manufacturer's instruction.

Two kinds of probes were used to identify GLRaV-2 specific clones from the library. One type was prepared from the synthesized cDNA that was amplified by PCR after ligation to the specific EcoR I Uni-Amp™ adapters (Clontech); and the other type was DNA inserts or PCR products from already sequenced clones. Clones from the cDNA library were selected by colony-lifting hybridization onto the colony/plaque Screen membrane (NEN Research Product) with the probe described above. The probe was prepared by labeling with <sup>32</sup>P [α-dATP] using Klenow fragment of *E.coli* DNA polymerase I. Prehybridization, hybridization, and washing steps were carried out at 65°C according to the manufacturer's instruction (Dupont, NEN Research Product). Selected plaques were converted to recombinant pBluescript by *in vivo* excision method according to the manufacturer's instruction (Stratagene).

To obtain clones representing the extreme 3'-terminus of GLRaV-2, dsRNA was polyadenylated by yeast poly(A) polymerase. Using poly(A)-tailed dsRNA as template, cDNA was amplified by RT-PCR with oligo(dT)18 and a specific primer, CP-1/T7R, which

is derived from the clone CP-1 and has a nucleotide sequence according to SEQ. ID. No. 20 as follows:

TGCTGGAGCT TGAGGTTCTG C

21

5

The resulting PCR product (3'-PCR) was cloned into a TA vector (Invitrogen) and sequenced.

As shown in Figure 1A, a high molecular weight dsRNA of ca. 15 kb was consistently identified from GLRaV-2 infected grapevines, but not from healthy vines. In addition, several low molecular weight dsRNAs were also detected from infected tissue. The yield of dsRNA of GLRaV-2 was estimated between 5-10 ng/15 g phloem tissue, which was much lower than that of GLRaV-3 (Hu et al., "Characterization of Closterovirus-Like Particles Associated with Grapevine Leafroll Disease," *J. Phytopathology* 128:1-14 (1990), which is hereby incorporated by reference). Only the high molecular weight dsRNA that was purified from low melting point agarose gel was used for cDNA synthesis, cloning and establishment of the Lambda/ZAP II cDNA library.

Two kinds of probes were used for screening the cDNA library. The initial clones were identified by hybridization with Uni-Amp™ PCR-amplified cDNA as probes. The specificity of these clones (e.g., TC-1) ranging from 200 to 1,800 bp in size was confirmed by Northern hybridization to dsRNA of GLRaV-2 as shown in Figure 1B. Additionally, over 40 different clones ranging from 800 to 7,500 bp in size were identified following hybridization with the probes generated from GLRaV-2 specific cDNA clones or from PCR products. Over 40 clones were then sequenced on the both strands (Figure 2).

#### 25 **Example 5 - Expression of the Coat Protein in *E. coli* and Immunoblotting**

To determine that ORF6 was the coat protein gene of GLRaV-2, the complete ORF6 DNA molecule was subcloned from a PCR product and inserted into the fusion protein expression vector pMAL-C2 (New England Biolabs, Inc.). The specific primers used for the PCR reaction were CP-96F and CP-96R, in which an EcoR I or BamH I site was included to facilitate cloning. CP-96F was designed to include the start codon of the CP and comprises a nucleotide sequence according to SEQ. ID. NO. 21 as follows:

CGGAATTAC CATGGAGTTG ATGTCCGACA G

31



CP-96R was 66 nucleotides downstream of the stop codon of the CP and comprises the nucleotide sequence corresponding to SEQ. ID. No. 22 as follows:

AGCGGATCCA TGGCAGATTC GTGCGTAGCA GTA

33

The coat protein was expressed as a fusion protein with maltose binding protein (MBP) of *E. coli* under the control of a "tac" promoter and suppressed by the "lac" repressor. The MBP-CP fusion protein was induced by adding 0.3 mM isopropyl- $\beta$ -D-thio-gloactopyranoside (IPTG) and purified by a one step affinity column according to the manufacturer's instruction (New England, Biolabs, Inc ). The MBP-CP fusion protein or the coat protein cleaved from the fusion protein was tested to react with specific antiserum of GLRaV-2 (kindly provided by Dr. Charles Greif of INRA, Colmar, France) on Western blot according to the method described by Hu et al., "Characterization of Closterovirus-Like Particles Associated with Grapevine Leafroll Disease," *J. Phytopathology* 128:1-14 (1990), which is hereby incorporated by reference. In contrast, the non-recombinant plasmids or uninduced cells did not react to the antiserum of GLRaV-2.

#### **Example 6 - Sequence Analysis and Genome Organization of GLRaV-2**

A total of 15,500 bp of the RNA genome of GLRaV-2 was sequenced and deposited in GenBank (accession number AF039204). About 85% of the total RNA genome was revealed from at least two different clones. The sequence in the coat protein gene region was determined and confirmed from several different overlapping clones. The genome organization of GLRaV-2, shown in Figure 2, includes nine open reading frames (e.g., ORF1a, 1b-8).

ORF1a and ORF1b: Analysis of the amino acid sequence of the N-terminal portion of GLRaV-2 ORF1a encoded product revealed two putative papain-like protease domains, which showed significant similarity to the papain-like leader protease of BYV (Agranovsky et al., "Beet Yellows Closterovirus: Complete Genome Structure and Identification of a Papain-like Thiol Protease," *Virology* 198:311-24 (1994), which is hereby incorporated by reference). Thus, it allowed prediction of the catalytic cysteine and histidine residues for the putative GLRaV-2 protease. Upon alignment of the sequence of the papain-like protease of BYV with that of GLRaV-2, the cleavage site at residues Gly-Gly (amino acid 588-589) of BYV aligned with the corresponding alanine-glycine (Ala-Gly) and Gly-Gly dipeptide of GLRaV-2 (Figure 3A). Cleavage at this site would result in a leader protein and

a 234 kDa (2090 amino acid) C-terminal fragment consisting of MT and HEL domains. However, the region upstream of the papain-like protease domain in GLRaV-2 did not show similarity to the corresponding region of BYV. In addition, variability in the residues located at the scissible bond (Gly in the BYV and Ala in the GLRaV-2) was present. Similar  
5 variability of the cleavage site residue in the P-PRO domain has been described in LChV (Jelkmann et al., "Complete Genome Structure and Phylogenetic Analysis of Little Cherry Virus, a Mealybug-Transmissible Closterovirus. J. General Virology 78:2067-71 (1997), which is hereby incorporated by reference).

Database searching with the deduced amino acid sequence of the ORF1a/1b  
10 encoded protein revealed a significant similarity to the MT, HEL and RdRP domains of the other closteroviruses. The region downstream of the P-PRO cleavage site showed a significant similarity (57.4% identity in a 266-residues alignment) to the putative methyltransferase domain of BYV and contained all the conserved motifs typical of positive-strand RNA viral type I MTs (Figure 3B). The C-terminal portion of the ORF1a was  
15 identified as a helicase domain, the sequence of which showed a high similarity (57.1% identity in a 315-residues alignment) to the helicase domain of BYV and contained the seven conserved motifs characteristic of the Superfamily I helicase of positive-strand RNA viruses (Figure 3C) (Hodgman, "A New Superfamily of Replicative Proteins," Nature 333:22-23 (1988); Koonin and Dolja, "Evolution and Taxonomy of Positive-strand RNA Viruses: Implications of Comparative Analysis of Amino Acid Sequences," Crit. Rev. in Biochem. and Mol. Biol. 28:375-430 (1993), both of which are hereby incorporated by reference).

ORF1b encoded a 460 amino acid polypeptide with a molecular mass of 52,486 Da, counting from the frameshifting site. Database searching with the RdRP showed a significant similarity to the RdRP domains of positive strand RNA viruses. Comparison of  
25 the RdRP domains of GLRaV-2 and BYV showed the presence of the eight conserved motifs of RdRP (Figure 3D).

As shown in Figure 8, a tentative phylogenetic tree of the RdRP of GLRaV-2 with respect to other closteroviruses shows that it is closely related to the monopartite closteroviruses BYV, BYSV, and CTV.

30 In closteroviruses, a +1 ribosomal frameshift mechanism has been suggested to be involved in the expression of ORF1b as a large fusion protein with ORF1a (Agranovsky et al., "Beet Yellows Closterovirus: Complete Genome Structure and Identification of a Papain-like Thiol Protease," Virology 198:311-24 (1994); Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," Virology 208:511-20 (1995); Klaassen

- et al., "Genome Structure and Phylogenetic Analysis of Lettuce Infectious Yellows Virus, a Whitefly-Transmitted, Bipartite Closterovirus," Virology 208:99-110 (1995); Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses," Virology 221:199-207 (1996); Jelkmann et al.,
- 5 "Complete Genome Structure and Phylogenetic Analysis of Little Cherry Virus, a Mealybug-Transmissible Closterovirus," J. General Virology 78:2067-71 (1997), all of which are hereby incorporated by reference). In the overlapping ORF1a/1b region of BYV, the slippery sequence of GGGUUUA and two hairpins structure (stem-loop and pseudoknot) are believed to result in a +1 frameshift (Agranovsky et al., "Beet Yellows Closterovirus: Complete
- 10 Genome Structure and Identification of a Papain-like Thiol Protease," Virology 198:311-24 (1994), which is hereby incorporated by reference). None of these features are conserved in CTV and BYSV (Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," Virology 208:511-20 (1995); Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses,"
- 15 Virology 221:199-207 (1996), both of which are hereby incorporated by reference), in which a ribosomal pausing at a terminator or at a rare codon was suggested to perform the same function. Comparisons of the nucleotide sequence of the C-terminal region of the helicase and the N-terminal region of RdRP of GLRaV-2 with the same region of other closteroviruses revealed a significant similarity to BYV, BYSV, and CTV. As shown in
- 20 Figure 4, the terminator UAG at the end of C'-terminal helicase of GLRaV-2 aligned with the terminator UAG of BYV and BYSV, and arginine CGG codon of CTV.

ORF2 encodes a small protein consisting of 171 bp (57 amino acid) with a molecular mass of 6,297 Da. As predicted, the deduced amino acid sequence includes a stretch of nonpolar amino acids, which is presumed to form a transmembrane helix. A small

25 hydrophobic analogous protein is also present in BYV, BYSV, CTV, LIYV, and LChV (Agranovsky et al. "Nucleotide Sequence of the 3'-Terminal Half of Beet Yellows Closterovirus RNA Genome Unique Arrangement of Eight Virus Genes," J. General Virology 72:15-24 (1991); Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses," Virology

30 221:199-207 (1996); Pappu et al., "Nucleotide Sequence and Organization of Eight 3' Open Reading Frames of the Citrus Tristeza Closterovirus Genome," Virology 199:35-46 (1994); Klaassen et al., "Partial Characterization of the Lettuce Infectious Yellows Virus Genomic RNAs, Identification of the Coat Protein Gene and Comparison of its Amino Acid Sequence With Those of Other Filamentous RNA Plant Viruses," J. General Virology 75:1525-33

(1994); Jelkmann et al., "Complete Genome Structure and Phylogenetic Analysis of Little Cherry Virus, a Mealybug-Transmissible Closterovirus," J. General Virology 78:2067-71 (1997), all of which are hereby incorporated by reference).

ORF3 encodes a 600 amino acid polypeptide with a molecular mass of 65,111 Da, which is homologous to the HSP70 cellular heat shock protein. HSP70 is highly conserved among closteroviruses and is probably involved in ATPase activity and the protein to protein interaction for chaperone activity (Agranovsky et al. "The Beet Yellows Closterovirus p65 Homologue of HSP70 Chaperones has ATPase Activity Associated with its Conserved N-terminal Domain but Interact with Unfolded Protein Chains," J. General Virology 78:535-42 (1997); Agranovsky et al., "Bacterial Expression and Some Properties of the p65, a Homologue of Cell Heat Shock Protein HSP70 Encoded in RNA Genome of Beet Yellows Closterovirus," Doklady Akademii Nauk. 340:416-18 (1995); Karasev et al., "HSP70-Related 65-kDa Protein of Beet Yellows Closterovirus is a Microtubule-Binding Protein," FEBS Letters 304:12-14 (1992), all of which are hereby incorporated by reference). As shown in Figure 5, alignment of the complete ORF3 of GLRaV-2 with HSP70 homolog of BYV revealed the presence of the eight conserved motifs. The percentage similarity of the HSP70 between GLRaV-2 and that of BYV, BYSV, CTV, LIYV, and LChV is 47.8%, 47.2%, 38.6%, 20.9%, and 17.7%, respectively.

ORF4 encodes a 551 amino acid protein with a molecular mass of 63,349 Da. Database searching with the ORF4 protein product did not identify similar proteins except those of its counterparts in closteroviruses, BYV (P64), BYSV (P61), CTV (P61), LIYV (P59), and LChV (P61). This protein is believed to be a putative heat shock 90 protein. As shown in Figure 9, two conserved motifs which were present in BYV (Agranovsky et al. "Nucleotide Sequence of the 3'-Terminal Half of Beet Yellows Closterovirus RNA Genome Unique Arrangement of Eight Virus Genes," J. General Virology 72:15-24 (1991), which is hereby incorporated by reference) and CTV (Pappu et al., "Nucleotide Sequence and Organization of Eight 3' Open Reading Frames of the Citrus Tristeza Closterovirus Genome," Virology 199:35-46 (1994), which is hereby incorporated by reference) were also identified in the ORF4 of GLRaV-2.

ORF5 and ORF6 encode polypeptides with molecular mass of 24,803 Da and 21,661 Da, respectively. The start codon for both ORFs is in a favorable context for translation. ORF6 was identified as the coat protein gene of GLRaV-2 based on the sequence comparison with other closteroviruses. The calculated molecular mass of the protein product of ORF6 (21,662 Da) is in good agreement with the previously estimated 22~26 kDa based

on SDS-PAGE (Zimmermann et al., "Characterization and Serological Detection of Four Closterovirus-like Particles Associated with Leafroll Disease on Grapevine," J. Phytopathology 130:205-18 (1990); Boscia et al., "Nomenclature of Grapevine Leafroll-Associated Putative Closteroviruses," Vitis 34:171-75 (1995), both of which are hereby  
5 incorporated by reference).

Database searching with the deduced amino acid sequence of the ORF6 of GLRaV-2 showed a similarity with the coat proteins of closteroviruses, BYV, BYSV, CTV, LIYV, LChV, and GLRaV-3. At the nucleotide level, the highest percentage similarity was with the coat protein of BYSV (34.8%); at the amino acid level, the highest percentage  
10 similarity was with the coat proteins of BYV (32.7%) and BYSV (32.7%). As shown in Figure 6A, alignment of the amino acid sequence of the coat protein and coat protein duplicate of GLRaV-2 with respect to other closteroviruses revealed that the invariant amino acid residues (N. R. G. D.) were present in both ORF5 and ORF6 of GLRaV-2. Two of these amino acid residues (R and D) are believed to be involved in stabilization of molecules  
15 by salt bridge formation and proper folding in the most conserved core region of coat proteins of all filamentous plant viruses (Dolja et al., "Phylogeny of Capsid Proteins of Rod-Shaped and Filamentous RNA Plant Viruses Two Families With Distinct Patterns of Sequence and Probably Structure Conservation," Virology 184:79-86 (1991), which is hereby incorporated by reference).

Identification of ORF6 as the coat protein gene was further confirmed by Western blot following expression of a fusion protein, consisting of a 22 kDa of ORF6 CP and a 42 kDa of maltose binding protein, produced by transformed *E. coli* as described in Example 5 *supra*. As shown in Figure 6B, the putative phylogenetic tree of the coat protein and coat protein duplicate of GLRaV-2 with those of other closteroviruses showed that  
20 GLRaV-2 is more closely related to aphid transmissible closteroviruses (BYV, BYSV, and CTV) (Candresse, "Closteroviruses and Clostero-like Elongated Plant Viruses," in Encyclopedia of Virology, pp. 242-48, Webster and Granoff, eds., Academic Press, New York (1994), which is hereby incorporated by reference) than to whitefly (LIYV) or mealybug transmissible closteroviruses (LChV and GLRaV-3) (Raine et al., "Transmission  
25 of the Agent Causing Little Cherry Disease by the Apple Mealybug *Phenacoccus aceris* and the Dodder *Cuscuta Lupuliformis*," Canadian J. Plant Pathology 8:6-11 (1986); Jelkmann et al., "Complete Genome Structure and Phylogenetic Analysis of Little Cherry Virus, a Mealybug-Transmissible Closterovirus," J. General Virology 78:2067-71 (1997); Rosciglione and Gugerli, "Transmission of Grapevine Leafroll Disease and an Associated Closterovirus  
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to Healthy Grapevine by the Mealybug *Planococcus ficus*," *Phytoparasitica* 17:63 (1989); Engelbrecht and Kasdorf, "Transmission of Grapevine Leafroll Disease and Associated Closteroviruses by the Vine Mealybug *planococcus-ficus*," *Phytophactica*, 22:341-46 (1990); Cabaleiro and Segura, 1997; Petersen and Charles, "Transmission of Grapevine

- 5 Leafroll-Associated Closteroviruses by *Pseudococcus longispinus* and *P. calceolariae*. *Plant Pathology* 46:509-15 (1997), all of which are hereby incorporated by reference).

ORF7 and ORF8 encode polypeptides of 162 amino acid with a molecular mass of 18,800 Da and of 206 amino acid with a molecular mass of 23,659 Da, respectively. Database searching with the ORF7 and ORF8 showed no significant similarity with any other  
10 proteins. Nevertheless, these genes were of similar in size and location as those observed in the sequence of other closteroviruses, BYV (P20, P21), BYSV (P18, P22), and LChV (P21, P27) (Figure 7). However, conserved regions were not observed between the ORF7 or ORF8 and its counterparts in BYV, BYSV, and LChV.

- The 3' terminal untranslated region (3'-UTR) consists of 216 nucleotides.  
15 Nucleotide sequence analysis revealed a long oligo(A) tract close to the end of the GLRaV-2 genome which is similar to that observed in the genome of BYV and BYSV (Agranovsky et al. "Nucleotide Sequence of the 3'-Terminal Half of Beet Yellows Closterovirus RNA Genome Unique Arrangement of Eight Virus Genes," *J. General Virology* 72:15-24 (1991); Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome  
20 and Implications for the Evolution of Closteroviruses," *Virology* 221:199-207 (1996), both of which are hereby incorporated by reference). The genome of BYV ends in CCC, BYSV, and CTV ends in CC with an additional G or A in the double-stranded replicative form of BYSV (Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses," *Virology* 221:199-207 (1996), which  
25 is hereby incorporated by reference) and CTV (Karasev et al., "Complete Sequence of the Citrus Tristeza Virus RNA Genome," *Virology* 208:511-20 (1995), which is hereby incorporated by reference), respectively . GLRaV-2 had CGC at the 3' terminus of the genome. Recently, a conserved 60 nt cis-element was identified in the 3'-UTR of three monopartite closteroviruses, which included a prominent conserved stem and loop structure  
30 (Karasev et al., 1996). As shown in Figure 10, alignment of the 3'-UTR sequence of GLRaV-2 with the same regions of BYV, BYSV, and CTV showed the presence of the same conserved 60 nt stretch. Besides this cis-element, conserved sequences were not found in the 3' UTRs of GLRaV-2, BYV, BYSV, and CTV.

The closteroviruses studied so far (e.g., BYV, BYSV, CTV, LIYV, LChV, and GLRaV-3) have apparent similarities in genome organization, which include replication associated genes that consist of MT, HEL, and RdRP conserved domains and a five-gene array unique for closteroviruses (Dolja et al. "Molecular Biology and Evolution of Closteroviruses: Sophisticated Build-up of Large RNA Genomes," Annual Rev. Photopathology 32:261-85 (1994); Agranovsky "Principles of Molecular Organization, Expression, and Evolution of Closteroviruses: Over the Barriers," Adv. in Virus Res. 47:119-218 (1996); Jelkmann et al., "Complete Genome Structure and Phylogenetic Analysis of Little Cherry Virus, a Mealybug-Transmissible Closterovirus," J. General Virology 78:2067-71 (1997); Ling et al., "Nucleotide Sequence of the 3' Terminal Two-Thirds of the Grapevine Leafroll Associated Virus-3 Genome Reveals a Typical Monopartite Closterovirus," J. General Virology 79(5):1289-1301 (1998), all of which are hereby incorporated by reference).

The above data clearly shows that GLRaV-2 is a closterovirus. In the genome of GLRaV-2, two putative papain-like proteases were identified and an autoproteolytic cleavage process was predicted. The replication associated proteins consisting of MT, HEL, and RdRP conserved motifs were also identified, which were phylogenetically closely related to the replication associated proteins of other closteroviruses. A unique gene array including a small hydrophobic transmembrane protein, HSP70 homolog, HSP90 homolog, diverged CP and CP was also preserved in GLRaV-2. In addition, the calculated molecular mass (21,661 Da) of the coat protein (ORF6) of GLRaV-2 is in good agreement with that of the other closteroviruses (22 to 28 kDa) (Martelli and Bar-Joseph, "Closteroviruses: Classification and Nomenclature of Viruses," Fifth Report of the International Committee on Taxonomy of Viruses, Francki et al., eds., Springer-Verlag Wein, New York, p. 345-47 (1991); Candresse and Martelli, "Genus *Closterovirus*," in Virus Taxonomy, Report of the International Committee on Taxonomy of Viruses, Murphy et al., eds., Springer-Verlag, NY, p. 461-63 (1995), both of which are hereby incorporated by reference). Two ORFs downstream of the CP are of similar, in size and location, to those observed in the genome of BYV. Furthermore, lack of a poly(A) tail at the 3' end of GLRaV-2 is also in good agreement with other closteroviruses. Like all other closteroviruses, the expression of ORF1b is suspected to occur via a +1 ribosomal frameshift and the 3' proximal ORFs are probably expressed via formation of a nested set of subgenomic RNAs. Since the slippery sequence, stem-loop and pseudoknot structure involved in the frameshift of BYV were absent in GLRaV-2, the +1 frameshift of GLRaV-2 might be the same as proposed for CTV (Karasev et al., "Complete

Sequence of the Citrus Tristeza Virus RNA Genome," Virology 208:511-20 (1995), which is hereby incorporated by reference) and BYSV (Karasev et al., "Organization of the 3'-Terminal Half of Beet Yellow Stunt Virus Genome and Implications for the Evolution of Closteroviruses," Virology 221:199-207 (1996), which is hereby incorporated by reference).

5 Overall, GLRaV-2 is more closely related to monopartite closteroviruses BYV, BYSV, and CTV than to GLRaV-3 (Figure 7) (Ling et al., "Nucleotide Sequence of the 3' Terminal Two-Thirds of the Grapevine Leafroll Associated Virus-3 Genome Reveals a Typical Monopartite Closterovirus," J. General Virology 79(5):1289-1301 (1998), which is hereby incorporated by reference), even though the latter causes similar leafroll symptoms in  
10 grapevine (Rosciiglione and Gugerli, "Maladies de l'Enroulement et du Bois Strie de la Vigne: Analyse Microscopique et Serologique (Leafroll and Stem Pitting of Grapevine: Microscopical and Serological Analysis)," Rev Suisse Viticult Arboricult Horticulture 18:207-11 (1986); Hu et al., "Characterization of Closterovirus-Like Particles Associated with Grapevine Leafroll Disease," J. Phytopathology 128:1-14 (1990), both of which are  
15 hereby incorporated by reference).

Closteroviruses are a diverse group with complex and heterogeneous genome organizations. So far, GLRaV-2 is the only closterovirus that matches with the genome organization of BYV, the type member of the genus *Closterovirus*. In addition, the genomic RNA of GLRaV-2 is about the same size as that of BYV; however, the transmission vector of  
20 GLRaV-2 is unknown. The genome organization of GLRaV-2 is more closely related to the aphid transmissible closteroviruses (BYV and CTV) than to whitefly (LIYV) or mealybug transmissible closteroviruses (LChV and GLRaV-3). Thus, it is possible that GLRaV-2 is transmitted by aphids. Aphid transmission experiments with GLRaV-2 should provide information that might help develop methods for further control of GLRaV-2.

25 A total of 15,500 nucleotides or over 95% of the estimated GLRaV-2 genome has been cloned and sequenced. GLRaV-2 and GLRaV-3 (Ling et al., "Nucleotide Sequence of the 3' Terminal Two-Thirds of the Grapevine Leafroll Associated Virus-3 Genome Reveals a Typical Monopartite Closterovirus," J. General Virology 79(5):1289-1301 (1998), which is hereby incorporated by reference) are the first grapevine leafroll associated  
30 closteroviruses that have been almost completely sequenced. The above data clearly justify the inclusion of GLRaV-2 into the genus *Closterovirus*. In addition, the information regarding the genome of GLRaV-2 would provide a better understanding of this and related GLRaVs, and add fundamental knowledge to the group of closteroviruses.



**Example 7 - Construction of the CP Gene of GLRaV-2 in Plant Expression Vector**

GLRaV-2 infected Vitis vinifera, cv Pinot Noir grapevines originated from a vineyard in central New York was used as the virus isolate, from which the cp gene of GLRaV-2 was identified. Based on the sequence information, two oligonucleotide primers have been designed. The sense primer CP-96F (SEQ. ID. No. 21) starts from the ATG initiation codon of the coat protein gene and the complementary primer CP-96R (SEQ. ID. No. 22) starts from 56 nucleotides downstream of the stop codon of the CP gene. A Nco I restriction site (11 bp in SEQ. ID. No. 21 and 13 bp in SEQ. ID. No. 22) is introduced in the beginning of both primers to facilitate the cloning. The coat protein gene of GLRaV-2 was amplified from dsRNA extracted from GLRaV-2 infected grapevine using reverse transcriptase polymerase chain reaction (RT-PCR). The PCR-amplified CP product was purified from low melting temperature agarose gel, digested with Nco I and cloned into the same enzyme digested plant expression vector pEPT8 (shown at Figure 11). After screening, the orientation of recombinant construct was checked by using the internal restriction site of the CP gene and directly sequencing the CP gene. The recombinant construct with translatable (sense) full length coat protein gene, pEPT8CP-GLRaV2, was going through for the further cloning. The plant expression cassette, which consisted of a double cauliflower mosaic virus (CaMV) 35S-enhancer, a CaMV 35S-promoter, an alfalfa mosaic virus (ALMV) RNA4 5' leader sequence, a coat protein gene of GLRaV-2 (CP-GLRaV-2), and a CaMV 35S 3' untranslated region as a terminator, was cut using the EcoR I restriction enzyme, isolated from low melting point temperature agarose gel, and cloned into the same restriction enzyme treated binary vector pGA482GG or pGA482G (a derivative of pGA482 (An et al., "Binary Vectors," in Plant Molecular Biology Manual, pp. A3:1-19, Gelvin and Schilperoot, eds., Kinwer Academic Publishers, Dordrecht, Netherlands (1988), which is hereby incorporated by reference). The resulting recombinants constructs are pGA482GG/EPT8CP-GLRaV2 (shown at Figure 11A), which contain both neomycin phosphotransferase (npt II) and  $\beta$ -glucuronidase (GUS) at the internal region of the T-DNA, and pGA482G/EPT8CP-GLRaV2 (shown at Figure 11B) without GUS. These recombinants constructs were separately introduced by electroporation into disarmed avirulent *Agrobacterium tumefaciens* strain C58Z707. The *Agrobacterium tumefaciens* containing the vector was used to infect *Nicotiana benthamiana* wounded leaf disks according to the procedure essentially described by Horsch et al., "A Simple and General Method for

Transferring Genes into Plants," Science 277:1229-1231 (1985), which is incorporated herein by reference.

**Example 8 - Analysis of Transgenic *Nicotiana benthamiana* Plants with the CP Gene of GLRaV-2**

NPT II-ELISA: Double-antibody sandwich enzyme linked immnuosorbent assay (DAS-ELISA) was used to detect the npt II enzyme with an NPT II-ELISA kit (5' prime to 3' prime, Inc., Boulder, Co.).

Indirect ELISA: Polyclonal antibodies to GLRaV-2, which were prepared from the coat protein expressed in *E. coli*, were used. Plates were coated with homogenized samples in extraction buffer (1:10, w/v) (phosphate buffered saline containing 0.05% Tween 20 and 2% polyvinyl pyrrolidone) and incubated overnight at 4°C. After washing with phosphate buffered saline containing 0.05% Tween 20 (PBST), the plates were blocked with blocking buffer (phosphate buffered saline containing 2% BSA) and incubated at room temperature for 1 hr. The anti-GLRaV-2 IgG was added at 2 µg/ml after washing with PBST. After incubation at 30 C for 4 hr, the plates were washed with PBST, and the goat anti-rabbit IgG conjugate of alkaline phosphatase (Sigma) was added at 1:10,000 dilution. The absorbance was measured at 405 nm with a MicroELISA AutoReader. In addition, Western blot was also performed according to the method described by Hu et al., "Characterization of Closterovirus-like Particle Associated Grapevine Leafroll Disease," J. Phytophathology 128:1-14, (1990), which is incorporated herein by reference.

PCR analysis: Genomic DNA was extracted from leaves of putative transgenic and non-transgenic plants according to the method described by Cheung et al., "A Simple and Rapid DNA Microextraction Method for Plants, Animal, and Insect Suitable for RAPD and other PCR analysis," PCR Methods and Applications 3:69 (1996), which is incorporated herein by reference. The extracted total DNA served as the template for PCR reaction. The primers CP-96F and CP-96R (SEQ. ID. Nos. 21 and 22, respectively) for the CP gene of GLRaV-2, as well as npt II 5'- and 3'- primers were used for PCR analysis. PCR reaction was performed at the 94°C x 3 min for one cycle, followed by 30 cycles of 94° C x 1 min, 50° C x 1 min, and 72° C x 2:30 min with an additional extension at 72° C for 10 min. The PCR product was analyzed on agarose gel.

After transformation, a total of 42 kanamycin resistant *Nicotiana benthamiana* lines (R<sub>0</sub>) were obtained, of which the leaf samples were tested by NPT II enzyme activity.

Among them, 37 lines were NPT II positive by ELISA, which took about 88.0% of total transformants. However, some of NPT II negative plants were obtained among these selected kanamycin resistant plants. All of the transgenic plants were self-pollinated in a greenhouse, and the seeds from these transgenic lines were germinated for further analysis.

5           The production of GLRaV-2 CP in transgenic plants was detected by indirect ELISA prior to inoculation, and the results showed that GLRaV-2 CP gene expression was not detectable in all transgenic plants tested. This result was further confirmed with Western blot. Using the antibody to GLRaV-2, the production of the CP was not detected in the transgenic and nontransgenic control plants. However, a protein of expected size (~22 kDa)  
10       was detected in GLRaV-2 infected positive control plants. This result was consistent with the ELISA result. The presence of the CP gene of GLRaV-2 in transgenic plants was detected from total genomic DNA extracted from plants tissue by PCR analysis (Figure 12). The DNA product of expected size (653 bp) was amplified from twenty tested transgenic lines, but not in non-transgenic plants. The result indicated that the CP gene of GLRaV-2 was present at  
15       these transgenic lines, which was also confirmed by Northern blot analysis.

**Example 9 - R<sub>1</sub> and R<sub>2</sub> transgenic *Nicotiana benthamiana* Plants Are Resistant to GLRaV-2**

20           Inoculation of transgenic plants: GLRaV-2 isolate 94/970, which was originally identified and transmitted from grapevine to *Nicotiana benthamiana* in South Africa (Goszczynski et al., "Detection of Two Strains of Grapevine Leafroll-Associated Virus 2," *Vitis* 35:133-35 (1996), which is incorporated herein by reference), was used as inoculum. The CP gene of isolate 94/970 was sequenced; and it is identical to the CP gene  
25       used in construction. *Nicotiana benthamiana* is an experimental host of GLRaV-2. The infection on it produces chlorotic and occasional necrotic lesions followed by systemic vein clearing. The vein clearing results in vein necrosis. Eventually the infected plants died, starting from the top to the bottom.

          At five to seven leaf stage, two youngest apical leaves were challenged with  
30       GLRaV-2 isolate 94/970. Inoculum was prepared by grinding 1.0 g GLRaV-2 infected *Nicotiana benthamiana* leaf tissue in 5 ml of phosphate buffer (0.01M K<sub>2</sub>HPO<sub>4</sub>, PH7.0). The tested plants were dusted with carborundum and rubbed with the prepared inoculum. Non-transformed plants were simultaneously inoculated as above. The plants were observed for symptom development every other day for 60 days after inoculation. Resistant R<sub>1</sub> transgenic

plants were carried on to R2 generation for further evaluation.

Transgenic progenies from 20 R<sub>0</sub> lines were initially screened for the resistance to GLRaV-2 followed by inoculation with GLRaV-2 isolate 94/970. The seedlings of the transgenic plants (NPT II positive), and nontransformed control plants were inoculated with GLRaV-2. After inoculation, the reaction of tested plants were divided into three types: highly susceptible (i.e. typical symptoms were observed two to four weeks postinoculation); tolerant (i.e. no symptom was developed in the early stage and typical symptoms was shown four to eight weeks postinoculation); and resistant (i.e. the plants remained asymptomatic eight weeks postinoculation). Based on the plant reaction, the resistant plants were obtained from fourteen different lines (listed in Table 1 below). In each of these fourteen lines, there was no virus detected within these plants by ELISA at 6 weeks postinoculation. In contrast, GLRaV-2 was detected in symptomatic plants by indirect ELISA. In the other six lines, although there were a few plants with some kind of delay in symptom development, all the inoculated transgenic plants died at three to eight weeks postinoculation. Based on the initial screening results, five representative lines consisting of three resistant lines (1, 4, and 19) and two susceptible lines (12 and 13) were selected for the further analysis.

Table 1

No. Line	No.	Reaction of Tested Plants		
		HS	T	HR
line 1	39	14	3	22
line 2	36	7	6	23
line 3	38	11	4	23
line 4	31	4	5	22
line 5	33	6	13	14
line 6	36	4	16	16
line 7	32	5	9	18
line 8	37	22	9	6
line 9	36	9	12	15
line 10	14	13	1	0
line 11	13	11	2	0
line 12	17	16	1	0
line 13	16	14	0	0
line 14	17	17	0	0
line 15	32	30	2	0
line 16	33	6	13	14
line 17	12	0	1	11
line 19	15	0	0	15
line 20	19	3	0	16
line 21	14	1	3	10
control	15	15	0	0

Table 1

		Reaction of Tested Plants		
No. Line	No.	HS	T	HR
No Line: include transgenic lines and nontransformed control;				
No: the number of transgenic and nontransformed plants;				
HS: highly susceptible, typical symptoms were observed two to four weeks after inoculation;				
T: tolerant, the symptoms were observed five to eight weeks after inoculation; and				
HR: plants remain without asymptoms after eight weeks inoculation.				

Table 2 below shows the symptom development in transgenic plants relative to non-transgenic control plants in the five selected lines in separate experiments. Non-transgenic control plants were all infected two to four weeks after inoculation, which showed typical GLRaV-2 symptoms on *Nicotiana benthamiana*, including chlorotic and local lesions followed by systemic vein clearing and vein necrosis on the leaves. Three of the tested lines (1, 4, and 19) showed some resistance that was manifested by either an absence or a delay in symptom development. Two other lines, 12 and 13, developed symptoms at nearly the same time as the non-transformed control plants. From top to bottom, the leaves of infected plants gradually became yellow, wilted, and dried, and, eventually, the whole plants died. No matter when infection occurred, the eventual result was the same. Six weeks after inoculation, all non-transgenic plants and the susceptible plants were dead. Some tolerant plants started to die. In contrast, the asymptomatic plants were flowering normally and pollinating as the non-inoculated healthy control plants (Figure 13).

Table 2

No. Line	No.	Reaction of Tested Plants		
		HS	T	HR
line 1	19	5	6	8
line 4	15	9	1	5
line 12	16	14	2	0
line 13	18	13	5	0
line 19	13	10	0	3
non-transgenic	24	23	1	0
No. Line: include transgenic lines and nontransformed control;				
No.: Number of transgenic and nontransformed plants tested;				
HS: highly susceptible; typical symptoms were observed two to four weeks after inoculation;				
T: tolerant, the symptoms were observed five to eight weeks postinoculation; and				
HR: plants remain without asymptoms after eight weeks inoculation.				

ELISA was performed at 6 weeks postinoculation to test the GLRaV-2 replication in the plants. Presumably, the increased level of CP reflected virus replication. The result showed that the absorbance value in symptomatic plants reached (OD) 0.7 to 3.2,

compared to (OD) 0.10-0.13 prior to inoculation. In contrast, GLRaV-2 was not detected in asymptomatic plants, of which the absorbance value was the same or nearly the same as that of healthy nontransformed control plants. The data confirmed that virus replicated in symptomatic plants, but not in asymptomatic plants. The replication of GLRaV-2 was suppressed in asymptomatic plants. This result implicated that another mechanism other than the CP-mediated resistance was probably involved.

Three R2 progenies derived from transgenic resistant plants of lines 1, 4, and 19 were generated and utilized to examine the stable transmission and whether resistance was maintained in R2 generation. These results are shown in Table 3 below. NPT II analysis revealed that R2 progeny were still segregating. The CP expression in R2 progeny was still undetectable. After inoculation, all the nontransgenic plants were infected and showed GLRaV-2 symptoms on the leaves after 24 days postinoculation. In contrast, the inoculated transgenic R<sub>2</sub> progeny showed different levels of resistance from those highly susceptible to highly resistant. The tolerant and resistant plants were manifested by a delay in symptom development and absence of symptoms, respectively. At 6 weeks postinoculation, GLRaV-2 was detected in the tolerant symptomatic infected plants by indirect ELISA; but not in asymptomatic plants. This result indicated that virus replication was suppressed in these resistant plants, which was confirmed by Western blot. These resistant plants remained asymptomatic eight weeks postinoculation, and they were flowering normally and pollinating.

Table 3

No. Line	No. Plants	NPT II positive/negative	HS	Reaction of Tested Plants	
				T	HR
line 1/22	12	12/20	3	3	6
line 1/30	11	8/3	7	2	2
line 1/31	11	10/1	6	3	2
line 1/35	10	10/0	4	6	0
line 1/41	8	7/1	2	2	4
line 4/139	12	11/1	4	4	3
line 4/149	10	7/3	4	5	1
line 4/152	10	8/2	9	0	1
line 4/174	9	8/1	4	0	4
line 19/650	11	10/1	7	0	2
line 19/657	12	12/0	6	2	4
line 19/659	12	8/4	5	2	5
line 19/660	10	8/2	3	6	1
non-transformed CK	12	0/12	12	0	0

HS: highly susceptible, typical symptoms were observed two to four weeks after inoculation;  
T: tolerant, the symptoms were observed five to eight weeks postinoculation; and  
HR: plants remain asymptomatic at eight weeks postinoculation.

**Example 10 - Evidence for RNA-Mediated Protection in Transgenic Plants**

Northern blot analysis: Total RNA was extracted from leaves prior to inoculation following the method described by Napoli et al., Plant Cell 2:279-89 (1990), which is hereby incorporated by reference. The concentration of the extracted RNA was measured by spectrophotometer at OD 260. About 10 g of total RNA was used for each sample. The probe used was the 3' one third of GLRaV-2 CP gene, which was randomly labeled with <sup>32</sup>P (α-dATP) using Klenow fragment of DNA polymerase I.

Using a DNA corresponding to the 3' one third CP gene sequence as probe, a single band was detected in the RNA extracted from susceptible plants from R1 progeny of lines 5, 12, and 13 by Northern hybridization. There was little or no signal detected in the transgenic plants from R1 progeny of line 1, 4, and 19. This RNA is not present in nontransformed control plants. The size of the hybridization signal was estimated to an approximately 0.9 kb nucleic acid, which was about the same as estimated (Figure 14). In lines of 1, 4, and 19, the steady state level of RNA expression was also low in R2 progeny. This data showed that susceptible plants from lines 12 and 13 had high mRNA level and all transgenic plants from lines 1, 4, and 19 had low mRNA level.

**Example 11 - Transformation and Analysis of Transgenic Grapevines with the CP Gene of GLRaV-2**

Plant materials: The rootstock cultivars Couderc 3309 (3309C) (*V. riparia* x *V. rupestris*), *Vitis riparia* 'Gloire de Montpellier' (Gloire), Teleki 5C (5C) (*V. berlandieri* x *V. riparia*), Millardet et De Grasset 101-14 (101-14 MGT) (*V. riparia* x *V. rupestris*), and Richter 110 (110R) (*V. rupestris* x *V. berlandieri*) were utilized. Initial embryogenic calli of Gloire were provided by Mozsar and Süle (Plant Protection Institute, Hungarian Academy of Science, Budapest). All other plant materials came from a vineyard at the New York State Agricultural Experiment Station, Geneva, NY. Buds were removed from the clusters and surface sterilized in 70% ethanol for 1-2 min. The buds (from the greenhouse and the field) were transferred to 1% sodium hypochlorite for 15 min, then rinsed three times in sterile, double-distilled water. Anthers were excised aseptically from flower buds with the aid of a stereo microscope. The pollen was crushed on a microscope slide under a coverslip with a drop of acetocarmine to observe the cytological stage. This was done to determine which stage was most favorable for callus induction.

Somatic embryogenesis and regeneration: Anthers were plated under aseptic conditions at a density of 40 to 50 per 9 cm diameter Petri dish containing MSE. Plates were cultured at 28°C in the dark. Callus was initiated, and, after 60 days, embryos were induced and were transferred to hormone-free HMG medium for differentiation. Torpedo stage embryos were then transferred from HMG to MGC medium to promote embryo germination. Cultures were maintained in the dark at 26-28°C and transferred to fresh medium at 3-4 week intervals. Elongated embryos were transferred to rooting medium in baby food jars (5-8 embryos per jar). The embryos were grown in a tissue culture room at 25°C with a daily 16 h photoperiod (76 :mol. s) to induce shoot and root formation. After plants developed roots, they were transplanted to soil in the greenhouse.

Transformation: The protocols used for transformation were modified from those described by Scorza et.al., "Transformation of Grape (*Vitis vinifera* L.) Zygotic-derived Somatic Embryos and Regeneration of Transgenic Plants," Plant Cell Rpt. 14:589-92 (1995), which is hereby incorporated by reference. Overnight cultures of *Agrobacterium* strain C58Z707 or LBA4404 were grown in LB medium at 28°C in a shaking incubator. Bacteria were centrifuged for 5 min at 3000-5000 rpm and resuspended in MS liquid medium (OD 1.0 at A600 nm ). Calli with embryos were immersed in the bacterial suspension for 15-30 min, blotted dry, and transferred to HMG medium with or without acetosyringone (100 µM). Embryogenic calli were co-cultivated with the bacteria for 48 h in the dark at 28°C. Then, the plant material was washed in MS liquid plus cefotaxime (300 mg/ml) and carbenicillin (200 mg/ml) 2-3 times. To select transgenic embryos, the material was transferred to HMG medium containing either 20 or 40 mg/L kanamycin, 300 mg/L cefotaxime, and 200 mg/L carbenicillin. Alternatively, after co-cultivation, embryogenic calli were transferred to initiation MSE medium containing 25 mg/l kanamycin plus the same antibiotics listed above. All plant materials were incubated in continuous dark at 28°C. After growth on selection medium for 3 months, embryos were transferred to HMG or MGC without kanamycin to promote elongation of embryos. They were then transferred to rooting medium without antibiotics. Nontransformed calli were grown on the same media with and without kanamycin to verify the efficiency of the kanamycin selection process.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.